The Pennsylvania State University

The Graduate School

Intercollege Program in Plant Physiology

# FUNCTIONAL PROTEOMICS OF *ARABIDOPSIS THALIANA* GUARD CELLS UNCOVERS NEW STOMATAL SIGNALING PATHWAYS

A Dissertation in

Plant Physiology

by

Zhixin Zhao



Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2008

The dissertation of Zhixin Zhao was reviewed and approved* by the following:

Sarah M. Assmann
Waller Professor of Biology
Dissertation Advisor
Chair of Committee

Hong Ma
Distinguished Professor of Biology

Ming Tien
Professor of Biochemistry and Molecular Biology

Reka Albert
Associate Professor of Physics and Biology

Jiaxu Li
Special Member
Assistant Professor of Biochemistry and Molecular Biology in Mississippi State University

Teh-hui Kao
Professor of Biochemistry and Molecular Biology
Chair of the Intercollege Graduate Program in Plant Physiology

*Signatures are on file in the Graduate School

## ABSTRACT

The guard cell is a specialized cell type, located in the epidermes of higher plants. The guard cell has been used as a model system in plant cell biology for decades. Here, I isolated a total of $3x10^8$ guard cell protoplasts from 22,000 Arabidopsis plants and identified 1,764 unique proteins using three complementary proteomic methods: protein spot identification from broad and narrow pH range 2D gels, and 2D LC-MALDI MudPIT. Proteomic study suggested that myrosinase 1 (TGG1) is the most abundant protein in guard cells. TGG1 catalyzes the production of toxic isothiocyanates from glucosinolates, and the glucosinolate-myrosinase system is well known as a defense system against biotic invaders. Phenotypic analysis showed that *tgg1* mutants were hyposensitive to abscisic acid (ABA)-inhibition of guard cell inward $K^+$ channels and stomatal opening, revealing that the glucosinolate-myrosinase system is also central to abiotic stress responses. TopGO analysis of the identified guard cell proteome revealed that proteins involved in energy production were enriched in the GC proteome. I further characterized mutants lacking the glycolytic enzyme iPGM (2,3-biphosphoglycerate-independent phosphoglycerate mutase). *ipgm* mutants showed defects in stomatal movements, growth, and pollen production in our study. Our study demonstrates that proteomic studies can make powerful contributions to the identification of novel signaling pathways.

In addition to the guard cell proteomic study, I also compared the proteomic patterns of Col and *gpa1-4* guard cells with and without ABA treatment using iTARQ technology. The *gpa1-4* mutant, lacking the heterotrimeric G protein α subunit, shows hyposensitivity to ABA inhibition of inward $K^+$ channels and ABA inhibition of stomatal opening. This iTRAQ study showed that two and six proteins were significantly regulated by ABA in protein abundance in Col and *gpa1*-4 guard cells respectively, while the abundance of 18 proteins in guard cells was affected by mutation of *GPA1*. Novel signaling models were proposed on the basis of the iTRAQ results.

To study the correlation of the transcriptome and proteome in guard cells, we also pursued microarray experiments. Comparison of transcriptome to proteome revealed that

the correlation between mRNA and protein levels is poor in Arabidopsis guard cells, suggesting that the protein abundance in guard cells is not primarily regulated at the transcriptional level.

# TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## ACKNOWLEDGEMENTS

I would like to express my great thanks and appreciation to my advisor, Dr. Sarah M. Assmann, for her support, encouragement, endless patience and constructive cristicism. She showed me how to be an independent researcher and a scientific writer. Her enthusiasm in science inspired me to continue my work in this field. I would also like to thank, Dr. Hong Ma, Dr. Jiaxu Li, Dr. Reka Albert, Dr. Ming Tien, and Dr. Daniel Jones, for serving as my thesis committee, and giving advises on my research and thesis writing.

I am grateful to Dr. Bruce Stanley and his wife, Mrs. Anne Stanley, in the College of Medicine Mass Spectromety and Proteomics Facility, and Dr. Lian-Chao Li in the Proteomics and Mass Spectrometry Core Facility in Penn State University.

My special appreciation also goes to the past and present lab members in Dr. Assmann's Lab, Dr. Cha Young Kim, Dr. Jerome Bove, Dr. Laetitia Perfus, Dr. Lei Ding, Dr. Liumin Fan, Dr. Riki Kawaguchi, Dr. Sona Pandey, Dr. Sylvie Coursol, Dr. Jaebok Heo, Dr. Laura Ullrich, Dr. Wei Zhang, Anne Gibson, Liza Wilson, Sarah Nilson, Song Li, Tim Gookin, and William Monsell. Thank you all for teaching me how to live and work together as a team. I was lucky to meet you in Dr. Assmann's Lab in Penn State and will miss you all.

I would also like to thank my parents and brothers. It is impossible for me to come to this stage without their support. My special thanks also go to my husband, Xiyan, for his love, care and continuous support. I am also grateful to my little angel, my daughter Yoyo. Thank her for all the happiness she brought to my life.

# Chapter 1

## Introduction

There are multiple genes encoding α, β and γ subunits of the heterotrimeric G protein in mammalian cells. By contrast, G proteins in Arabidopsis have only one Gα (*GPA1*), one Gβ (*AGB1*) and two known Gγs genes (*AGG1* and *AGG2*). G proteins are implicated in a wide range of signaling pathways in Arabidopsis and have been reviewed recently (Assmann, 2004; Perfus-Barbeoch et al., 2004; Assmann, 2005; McCudden et al., 2005; Temple and Jones, 2007). Given the fact that mutants lacking the Gα subunit, *gpa1-1* and *gpa1-2*, in the WS background (Wang et al., 2001) and *gpa1-4* in the Col background (Fan et al., 2008) are insensitive to ABA inhibition of inward $K^+$ channels and stomatal opening, one objective of my thesis study is to elucidate how GPA1 works with other proteins in ABA regulation of stomatal movements in Arabidopsis. Proteomic technologies are applied to identify protein candidates in ABA regulation of the GPA1 signaling pathway in guard cells. Considering the importance of guard cell function in research and agricultural aspects, proteins expressed in Arabidopsis guard cells are also investigated via proteomic technologies.

In my thesis study, I use guard cells as plant materials, proteomic methods as the primary technologies, and I study GPA1 signaling in Arabidopsis. Therefore, in this chapter, I introduce three subjects: guard cells, G proteins and proteomic methodologies. First, I discuss the value of the guard cell as an experimental model for plant biologists, as well as ABA and light signal transduction in Arabidopsis guard cells. Then, I introduce the Arabidopsis G protein subunits, Gα, Gβ, and Gγs followed by a review of GPA1 functions in Arabidopsis and discovery of GPA1 interacting proteins. Finally, I describe proteomic technologies, especially quantitative proteomics using stable isotope tags and application of these techniques to Arabidopsis.

## Introduction to guard cells

Guard cells are specialized cells located in the epidermes of higher plants and develop from guard mother cells by symmetric division. The number of guard cells per leaf is affected by development, drought, and carbon dioxide ($CO_2$) concentration

(Casson and Gray, 2008). Each pair of guard cells delineates a microscopic pore called a stoma. The size of the stomatal pore is regulated by changes in turgor pressure and volume of guard cells in response to various hormones and environmental cues, including abscisic acid (ABA), drought, humidity, $CO_2$, and light intensity (Fan et al., 2004).

The guard cell is a good experimental model to investigate signaling mechanisms in plants for several reasons. First, guard cells function as simple regulators of stomatal apertures, which are regulated by many types of stimuli such as light, $CO_2$, plant hormones, and environmental stresses. All these responses can be cell-autonomous in guard cells because isolated guard cells and guard cell protoplasts have been shown to respond to these stimuli. Most importantly, the consequences of these stimulants can be easily assessed by quantifying stomatal movements (opening or closing) in isolated epidermes under a microscope (Schroeder et al., 2001b). Second, guard cell protoplasts can be isolated and patch-clamped to monitor ion fluxes across the plasma membrane, thus providing direct evidence for signaling events. Third, a slew of data indicate that fundamental signaling events, for example ABA signaling in guard cells, might share some common cellular components with other cell types (Ghelis et al., 2000). Therefore, as a unique plant cell model with advantages in experimental manipulation, guard cells have become a focus for researchers who study signal transduction mechanisms in plants. In this chapter, I focus on discussion of stomatal movements in response to ABA and light because the mutants identified in my guard cell proteomic study (see Chapter 2) show phenotypes under ABA and light stimulation.

**ABA signaling in guard cells**

ABA is one of the five major plant hormones and plays important roles in many aspects of plant growth and development, e.g. seed dormancy and germination, early seedling development and stomatal movements (Xie et al., 2005). Under stress conditions, especially under drought stress, plants enhance their ABA content through ABA biosynthesis to facilitate their acclimation to environment changes (Seo and

Koshiba, 2002). ABA is perceived by ABA receptors. To date, three ABA receptors have been reported, including an RNA-binding protein (FCA) (Razem et al., 2006), the H subunit of Mg-chelatase (CHLH renamed as ABAR) (Shen et al., 2006), and a G protein-coupled receptor (GCR2) (Liu et al., 2007). FCA and CHLH share some features as ABA receptors: 1) Both FCA and CHLH had other known functions before their roles as ABA receptors were reported. FCA is an RNA-binding protein and acts as an transcriptional regulator of flowering time in Arabidopsis (Macknight et al., 1997); CHLH is a subunit of the Mg-chelatase involved in photosynthetic apparatus production and plastid-to-nucleus signaling pathway (Mochizuki et al., 2001). 2) Both FCA and CHLH were revealed to be possible ABA receptors because of their homology to affinity-purified ABA binding proteins. FCA shares protein sequence similarity with ABAP1. ABAP1 was isolated via screening of barley cDNA library using polyclonal anti-idiotypic antibodies (AB2) and binds to ABA *in vitro* (Razem et al., 2004); cDNA encoding the C-terminus of CHLH (ABAR) was cloned based on sequencing information from an ABA-binding protein purified from *Vicia faba* via affinity column purification (Zhang et al., 2002). Biochemical and phenotypic analyses showed that FCA was involved in controlling flowering time and RNA processing (Razem et al., 2006), and CHLH (ABAR) was involved in several classical ABA-regulated processes, such as seed germination, early seedling development and stomatal movements (Shen et al., 2006). Conflicting results were reported about GCR2 as an ABA receptor. Phenotypic analyses of *gcr2* mutants showed that known ABA responses including seed dormancy and germination, early seedling development and stomatal movements were defective in *gcr2* mutants, and biochemical assay also showed that GCR2 binds to ABA specifically (Liu et al., 2007). The authors thus concluded that GCR2 is also an ABA receptor (Liu et al., 2007). However, a more recent study argued that GCR2 is not even required in ABA inhibition of seed germination and early seedling development by phenotypic analyses of *gcr2* and *gcl1* (GCL1 is a homolog of GCR2) mutants (Gao et al., 2007). Reasons for these conflicting results are still unknown.

Upon perception by its receptors, ABA initiates downstream signaling pathways. ABA promotes stomatal closure and inhibits stomatal opening by regulating turgor

pressure of guard cells (MacRobbie, 1998; Wang et al., 2001; Fan et al., 2004; Pandey and Assmann, 2004; Li et al., 2006). Ion fluxes (e.g., $K^+$, $Ca^{2+}$, $Cl^-$ and malate$^{2-}$) out and into guard cells induce the osmolarity alterations that underlie stomatal movements (Schroeder et al., 2001b; Fan et al., 2004; Pandey et al., 2007). $K^+$ plays a major role in regulation of stomatal movements. Voltage-dependent inward $K^+$ channels mediate $K^+$ influx (Ichida et al., 1997; Kwak et al., 2001) and can be inhibited by ABA (Schroeder et al., 1987; Schwartz et al., 1994; Wang et al., 2001). In contrast, release of $K^+$ from guard cells is controlled by outward $K^+$ channels and ABA promotion of outward $K^+$ currents was detected in *Vicia faba* guard cells (Blatt, 1990). However, enhancement of outward $K^+$ currents by ABA in Arabidopsis has not been reported.

Besides ions, proteins are also important components in ABA regulation of stomatal movements. With the advent of systems biology techniques, a comprehensive guard cell dynamic biological model was created, including 13 signal transduction proteins, based on published results concerning ABA induction of stomatal closure in Arabidopsis and fava bean (Li et al., 2006). Phenotypic analysis of stomatal movements under ABA treatment in mutants is a major way to identify new signaling proteins. Mutants with impaired ABA regulation of stomatal movements have been described in a previous review (Israelsson et al., 2006). To date, 24 proteins have been reported to be involved in ABA-regulated stomatal movements (**Table 1-1**). Among the 24 proteins, ten are either protein phosphatases or protein kinases (**Table 1-1**), indicating that phosphorylation and dephosphorylation are important post-translational modifications in ABA signaling in guard cells. Four proteins have been shown to interact with GPA1 experimentally (GCR1, GCR2, AGB1 and PLDα1) indicating that GPA1 is an important component in ABA signaling.

**Light promotes stomatal opening**

Light, particularly blue light and red light, promotes stomatal opening and ABA inhibits light-promotion of stomatal opening (Shimazaki et al., 2007). It was reported

recently that nitric oxide (NO) and hydrogen peroxide ($H_2O_2$), known as effectors in ABA regulated stomatal movements, can also inhibit blue light promotion of stomatal opening via inhibition of the $H^+$-ATPase, the main membrane potential generator in plant cells (Zhang et al., 2007).

Two classes of blue light receptors have been identified: the cryptochromes and the phototropins (Briggs et al., 2001). The cryptochrome family regulates photomorphogenetic responses such as light inhibition of stem elongation and promotion of leaf expansion, and the phototropin family mediates phototropic responses. PHOT1 and PHOT2 are blue light receptors for phototropism in Arabidopsis and were identified to function in blue light promotion of stomatal opening by genetic analyses of *phot1*, *phot2* and *phot1 phot2* mutants (Kinoshita et al., 2001; Christie, 2007). Stomata in either *phot1* or *phot2* single mutant plants responded to blue light. However, this response was abolished in the *phot1 phot2* double mutant (Kinoshita et al., 2001). These data indicate a redundant blue light-perceiving function of PHOT1 and POHT2 in promotion of stomatal opening.

During blue light-induction of stomatal opening, the plasma membrane $H^+$-ATPase activity is up-regulated to modify the membrane potential, which in turn opens voltage-gated $K^+$ channels, resulting in $K^+$ accumulation in guard cells (Kinoshita and Shimazaki, 1999). To compensate for the positively charged $K^+$ ions, negatively charged $malate^{2-}$ and $Cl^-$ are preferentially accumulated in guard cells (Shimazaki et al., 2007). Malate can be produced by starch degradation through glycolysis. Phosphoglucomutase transfers a phosphoryl group between glucose 1-phosphate and glucose 6-phosphate upstream of the glycolytic pathway. Previous data showed that the blue light-induction of stomatal opening was impaired in a starch-deficient mutant, phosphoglucomutase mutant (Lasceve et al., 1997). This provides a rationale for studying roles of glycolysis in light induction of stomatal opening (Chapter 2).

**Introduction to heterotrimeric G protein**

G protein signaling mechanisms in animals are better understood than those in plants. Therefore, I borrow the basic concepts of animal G protein signaling here. G proteins are composed of α, β, and γ subunits. The Gα subunit has an α-helical domain affecting GDP release rate, and a GTPase domain with the ability to hydrolyze GTP. The GDP-bound G protein is inactive. Normally, the G protein can be activated by its G protein coupled receptor (GPCR), which is a plasma membrane protein with seven transmembrane domains. As a result of activation by the GPCR, the bound GDP is exchanged for GTP, and the G protein complex dissociates into the Gα subunit-GTP and the Gβγ subunit pair, either of which can induce downstream signaling by interacting with respective effectors. After the GTP is hydrolyzed to GDP by intrinsic GTPase activity of the Gα subunit, Gα re-associates with Gβγ to reform an inactive complex. During the whole process, the Gβγ subunit pair remains tightly associated. GPCRs, found in all eukaryotes, represent the largest family of cell surface receptors. The functional diversity of G protein complexes is supported by a great compositional variety of G proteins that can be made up of combinations of α, β, and γ subunits (about 23 Gα, 5 Gβ and 12 Gγ; >1380 combinations) in mammals (Assmann, 2004; McCudden et al., 2005). However, only one Gα (*GPA1*), one Gβ (*AGB1*) and two Gγ (*AGG1* and *AGG2*) subunits have been identified in Arabidopsis despite the fact that G proteins are implicated in multiple signaling pathways in plants (Assmann, 2002, 2004; Temple and Jones, 2007). It is therefore likely that plant G proteins have evolutionally adapted a different strategy in signal transduction than their animal counterparts. One hypothesis is that the signal specificity of G protein in plant cells is determined by down-stream signaling components, either directly or indirectly. To provide background for this perplexing question, here I emphasize the subunits and functions of G proteins in Arabidopsis with a focus on GPA1 interacting proteins.

**Identification of G protein subunits and physical interactions between G protein subunits**

All genes encoding G protein subunits have been identified in the completed Arabidopsis genome, including one α subunit (*GPA1*), one β subunit (*AGB1*), and two likely γ subunits (*AGG1* and *AGG2*). The first plant Gα subunit was cloned from Arabidopsis based on its homology to mammalian and yeast Gα proteins (Ma et al., 1990). Since then, Gα subunits have been cloned from many other plant species (Assmann, 2002). Plant Gαs share ~70-90% identity with each other; in contrast, the identity decreases to 34-42% when compared with nonplant homologs (Plakidou-Dymock et al., 1998). Gβ subunits were first cloned from Arabidopsis (*AGB1*) and maize (*ZGB1*) and share more than 41% identity with animal Gβ subunits (Weiss et al., 1994). The last G protein subunits to be identified, Gγs (*AGG1*, *AGG2*), were isolated from Arabidopsis by using a tobacco Gβ subunit as a bait protein in yeast two-hybrid screening (Mason and Botella, 2000, 2001).

Interactions between Gγ subunits (AGG1 and AGG2) and Gβ subunit (AGB1) were further confirmed by yeast two-hybrid and *in vitro* binding assays (Mason and Botella, 2000, 2001). Using fluorescence resonance energy transfer (FRET) technology, transiently expressed AGB1 was shown to form a heterodimer with either AGG1 or AGG2 at the plasma membrane in living cowpea mesophyll protoplasts. Lipidation of AGG1 and AGG2 are required to form the heterodimers (Adjobo-Hermans et al., 2006). Heterotrimer formation of GPA1-AGB1-AGG1 was also confirmed by FRET in both living cowpea and Arabidopsis mesophyll protoplasts (Adjobo-Hermans et al., 2006; Wang et al., 2008). Lipidation of GPA1 was shown to be required for heterotrimer formation in cowpea mesophyll protoplasts (Adjobo-Hermans et al., 2006). It was also reported that mutation of Cys to Ser in the isoprenylation motif disrupted the plasma membrane localization of AGG1 and AGG2 (Zeng et al., 2007) and lack of this motif returned the βγ dimer from plasma membrane to cytosol (Adjobo-Hermans et al., 2006). Experimental data from FRET and co-immunoprecipitation studies revealed that GPA1

interacts with the AGB1-AGG1 dimer at the plasma membrane and not with AGB1 or AGG1 alone in Arabidopsis (Wang et al., 2008). Results from blue native PAGE gel showed that about 30% of GPA1 and 100% of AGB1 were detected in large plasma membrane complexes (~ 700 kD). However, GPA1 forms several relatively smaller plasma membrane complexes (140 kD-400 kD) when AGB1 is absent (Wang et al., 2008). These results support that GPA1 interacts with AGB1 at the plasma membrane in Arabidopsis. Recently, the physical interaction of GPA1 with AGB1 in Arabidopsis suspension cells was confirmed via coimmuoprecipitation (Fan et al, 2008).

In mammalian systems, once Gα dissociates with Gβγ, both Gα and Gβγ can interact with downstream effectors. Some of these downstream effectors bind to Gα as well as Gβγ, and others only interact with either Gα or Gβγ (Temple and Jones, 2007). Interaction between Gα and Gβγ has been reported in Arabidopsis (Wang, et al., 2008), indicating that a similar mechanism may exist in Arabidopsis. If the heterotrimer Gαβγ is the functional unit, or Gα and Gβγ interact with the same downstream effectors, or Gα is the functional subunit but Gβγ subunit is required for plasma membrane localization of the Gα subunit, which was observed in mammalian cells (Fishburn et al., 2000; Evanko et al., 2001), then Arabidopsis mutants lacking either Gα or Gβ will have similar phenotypes. However, when Gβ is the main functional subunit, mutants lacking Gβ will have unique or stronger phenotypes compared to Gα. In next section, I summarize functions of G protein subunits obtained via phenotypic studies of Arabidopsis mutants lacking G protein subunit(s).

## Functions of G protein subunits in Arabidopsis

### Functions of Gα subunit

By screening T-DNA-tagged Arabidopsis mutants from the University of Wisconsin Arabidopsis knockout facility, two null alleles of the Gα subunit, *gpa1-1* and

*gpa1-2*, were found in the Arabidopsis WS ecotype background (Ullah et al., 2001). The T-DNA insertion is in the seventh intron in *gpa1-1* mutant, and in the eighth exon in *gpa1-2*. Two T-DNA insertional mutants in the Col ecotype background have also been reported: *gpa1-3* has an insertion in the ninth exon and *gpa1-4* has an insertion in the 12$^{th}$ intron (Jones et al., 2003). Seedlings from all the *gpa1* mutants show identical short hypocotyls and open hooks under darkness indicating functional similarity of GPA1 in WS and Col backgrounds (Jones et al., 2003). The *gpa1* mutants in WS background were shown to be protein null mutants (Ullah et al., 2001), and the *gpa1* mutants in Col background were suggested to be transcript null mutants (Jones et al., 2003). *gpa1-4* was also shown to be a null mutant at the protein level based on western analysis (Fan et al., 2008).

Cell division is reduced in both stems and leaves in *gpa1-1* and *gpa1-2* null mutants. Ectopic cell division, especially increased cell division in the shoot meristem, was found in inducible overexpression lines with high level of GPA1. These data suggest that GPA1 promotes cell division in Arabidopsis (Ullah et al., 2001).

Phenotypic studies show that *gpa1* (*gpa1-3* and *gpa1-4*) mutants are more sensitive to high concentration of D-glucose in inhibition of seed germination (Chen et al., 2006b) and early seedling growth (Huang et al., 2006) in Arabidopsis. Regulators of G protein signaling (RGS) accelerate GTP hydrolysis activity for Gα, thus rapidly switching the G protein from the active state to the inactive state (Hooks et al., 2008). AtRGS1 is an RGS in Arabidopsis with a putative N terminal seven transmembrane domain and a C terminal RGS box and interacts with GPA1 (Chen et al., 2003). Phenotypic analysis reveals that *rgs1-2* is hyposensitive to the arrested seedling development caused by high concentration of D-glucose (Johnston et al., 2007b), and transgenic plants overexpressing a constitutively active GPA1 have the same phenotype as that of *rgs1-2* (Huang et al., 2006). The *gpa1-4 rgs1-2* double mutant shows similar sensitivity to D-glucose in inhibition of seedling development as *gpa1-4* (Johnston et al., 2007b), and D-glucose enhances interaction between AtRGS1 and GPA1 in stable transgenic Arabidopsis plants coexpressing GPA1-CFP and AtRGS1-YFP (Johnston et al., 2007b). Importantly, the point mutation (E320K) in AtRGS1, which abolishes the

GTPase-accelerating activity, can disrupt the interaction between AtRGS1 and GPA1 (Johnston et al., 2007b). All these data led Johnson et al. (2007b) to conclude that AtRGS1 acts as a D-glucose receptor and regulates D-glucose signaling via affecting the hydrolysis of GPA1-GTP in Arabidopsis root development.

GPA1 also directly affects ABA signaling in guard cells (Wang et al., 2001). Anion channels in the plasma membrane of guard cells can be activated by ABA through two pathways, pH-dependent or pH-independent. Under either situation, anion efflux depolarizes the plasma membrane, leading to reduction of $K^+$ influx and promotion of $K^+$ efflux (Schroeder et al., 2001a; Wang et al., 2001). ABA-activation of the pH-independent pathway is abolished in *gpa1* guard cells, however the pH-dependent pathway is not affected in *gpa1* (Wang et al., 2001). These results suggest that GPA1 positively regulates ABA-inhibition of stomatal opening via the pH-independent pathway in Arabidopsis. The *gpa1* (*gpa1-1, gpa1-2* and *gpa1-4*) mutants are hyposensitive to ABA-inhibition of inward $K^+$ channels and stomatal opening in Arabidopsis (Wang et al., 2001; Fan et al., 2008). By contrast, ABA-promotion of stomatal closure is not affected by GPA1 (Wang et al., 2001). Outward $K^+$ channels in Arabidopsis are not affected by ABA in Col and the same lack of response to ABA is observed in the *gpa1-4* mutant (Fan et al., 2008).

Sphingosine-1-phosphate (S1P) is a ligand for mammalian GPCRs and has been reported to be formed in response to diverse stimuli in mammalian cells (Pyne and Pyne, 2000). In plants, S1P was shown to induce stomatal closure in *Commelina communis* epidermal strips (Ng et al., 2001). Since ABA enhances sphingosine kinase (SphK) activity, which is responsible for S1P production in Arabidopsis (Coursol et al., 2003), and ABA inhibition of stomatal opening was reduced in *gpa1* mutants (Wang et al., 2001), the effects of S1P on GPA1 signaling in guard cells were also studied (Coursol et al., 2003). Regulation of stomatal movements by S1P (both inhibition of opening and promotion of closure) is eliminated in *gpa1* mutants. By patch-clamping Arabidopsis guard cell protoplasts, S1P was shown to inhibit the inward $K^+$ channels and stimulate the slow anion channels in wild type cells. However these effects are abolished in *gpa1-1* and

*gpa1-2* mutants (Coursol et al., 2003) indicating that S1P positively regulates GPA1 in ABA signaling in Arabidopsis guard cells.

Besides the effects on ABA signaling in guard cells, GPA1 also affects ABA signaling in seed germination and early seedling development (Ullah et al., 2002; Pandey et al., 2006). Despite the hyposensitivity to ABA in guard cells, *gpa1* mutants are hypersensitive to ABA inhibition of seed germination and early seedling development. When seeds were germinated on plates supplemented with exogenous ABA, *gpa1-3* and *gpa1-4* mutant seeds had lower germination rates compared to wild type seeds (Pandey et al., 2006). It was proposed that AGB1 is the predominant regulator in ABA inhibition of seed germination (Pandey et al., 2006).Therefore, the effect of AGB1 on ABA inhibition of seed germination will be discussed in the next section.

**Functions of Gβ subunit**

Similar to the Gα subunit, phenotypic analyses of *agb1* null mutants, namely the point mutant *agb1-1* (Lease et al., 2001) and the T-DNA insertional mutant *agb1-2* (Ullah et al., 2003), have been widely undertaken. Three phenomena have been seen to date based on phenotypic comparisons between *gpa1* and *agb1* mutants: 1) *gpa1* and *agb1* mutants share similar mutant phenotypes; 2) *gpa1* and *agb1* mutants have different mutant phenotypes; 3) *agb1* mutants show mutant phenotypes but *gpa1* mutants show wild-type phenotypes

*gpa1 and agb1 share the same phenotypes*

Morphological analysis of *gpa1* (*gpa1-1* and *gap1-2*) and *agb1* mutant plants revealed that both *gpa1* and *agb1* mutants share some similar phenotypes, e. g. similarly rounded leaves, and greater fruit and seed weights compared to wild type (Ullah et al., 2003). The *agb1* mutants also show identical phenotypes as *gpa1* mutants in ABA

regulation of $K^+$ channels, anion channels and stomatal movements in Arabidopsis (Fan et al., 2008).

*gpa1 and agb1 show different phenotypes*

Morphological analysis also showed that *gpa1* and *agb1* mutants have different phenotypes in many aspects. Compared to wild type, *gpa1-2* and *agb1-2* have less and more root mass, smaller and larger seedlings, decreased and increased apical dominance, and longer and shorter sepals, respectively (Ullah et al., 2003). Treatment of *gpa1* and *agb1* mutants with exogenous auxin resulted in different responses in their lateral root growth. Both *agb1* mutants have more lateral roots than wild type whereas *gpa1-1* and *gpa1-2* mutants have less. This observation led Ullah et al. to speculate that Gβγ negatively regulates auxin-induced cell division in lateral roots (Ullah et al., 2003). Furthermore, another genetic study reported faster root growth rate and more lateral roots in the *agb1-2* mutant, and normal root growth rate and fewer lateral roots in *gpa1* mutants (*gpa1-3* and *gpa1-4*) (Chen et al., 2006a). In a proposed G protein working model in the Arabidopsis root, AGB1 negatively regulates lateral root formation and GPA1 positively regulates lateral root formation by inhibition of AGB1 through recruitment of AGB1 into the heterotrimer (Chen et al., 2006a).

All *gpa1* and *agb1* mutants were found to be hypersensitive to ABA-reduced seed germination. However, *agb1* showed a stronger phenotype than that of *gpa1* (*gpa1-3* and *gap1-4*), and the double mutants lacking both GPA1 and AGB1 showed a similarly strong phenotype as the *agb1* single mutant (Pandey et al., 2006). Given the fact that GPA1 protein is absent but *AGB1* transcript is present during germination if seeds were stratified, Pandey et al. proposed that AGB1 might be the functional unit in ABA inhibition of seed germination (Pandey et al., 2006).

G proteins were also suggested to be involved in multiple defense pathways in Arabidopsis. The *gpa1* and *agb1* plants are hyposensitive and hypersensitive to ozone ($O_3$) induced tissue damage respectively (Joo et al., 2005). When treated with *P.*

*cucumerina*, a pathogenic fungus, *gpa1-4* was more resistant and *agb1-1* was more susceptible (Llorente et al., 2005), and similar phenotypic results were obtained when *gpa1-4* and *agb1-2* mutant plants were treated with necrotrophic fungi (*F. oxysporum* and *A. brassicicola*) (Trusov et al., 2006). Importantly, the *gpa1-4 agb1-2* double mutant showed similar susceptible phenotypes as the *abg1-2* mutant to the necrotrophic fungi (*F. oxysporum* and *A. brassicicola*) (Trusov et al., 2006). These results further support that it is the β subunit, not the α subunit, that is primarily involved in defense pathways in Arabidopsis.

*agb1 mutants have mutant phenotypes but gpa1 mutants show wild type phenotypes*

AGB1 protein is degraded during the unfolded protein response (UPR), a protective response that is activated by the antibiotic tunicamycin (Tm) (Wang et al., 2007). The *agb1-2* plants are more resistant to Tm-inhibited seedling growth and Tm-induced cell death in mature leaves than *gpa1-4* and wild type, and the *gpa1-4 agb1-2* double mutant has similar sensitivity to Tm as *agb1-2* (Wang et al., 2007). Similarly, the $O_3$-induced leaf curling phenotype is only present in the *agb1-2* mutant but not in *gpa1-3* and *gpa1-4* mutants (Booker et al., 2004).

**Functions of Gγ subunits**

The isoprenylation motif in Gγs has been shown to be necessary for the plasma membrane localization of Gγs (Zeng et al., 2007) and the βγ dimer (Adjobo-Hermans et al., 2006). Transgenic plants overexpressing a truncated γ1 subunit lacking the C-terminal isoprenylation motif (γ*), which disrupts plasma membrane localization of αβγ trimer, were generated (Chakravorty and Botella, 2007). RT-PCR assay suggested that the transcript levels of the wild type G protein subunits (*GPA1*, *AGB1*, *AGG1* and *AGG2*) in the 35s-γ* transgenic plants were comparable to that in wild type plants, however,

northern results showed that the transcript level of γ* was much higher than that of wild type *AGG1*. 35s- γ* plants had similar but less severe plant morphological phenotypes as *agb1-2* mutants including the same reduced hypocotyl hook angle phenotype in the presence of 1-amino-cyclopropane-1-carboxylic acid (ACC) as *gpa1-4* and *agb1-2* (Chakravorty and Botella, 2007). These results indicate functional association of GPA1 and AGB1 with AGG1 in ABA and ethylene signaling.

Consistent with the traditional working model of heterotrimer G proteins in mammalian cells, the expression patterns of AGG1 and AGG2 resemble the expression pattern of AGB1 in most tissues except in flowers and siliques in Arabidopsis, indicating functional association of Gβγ subunits (Trusov et al., 2007; Trusov et al., 2008). Both T-DNA insertional *agg* mutants (*agg1-1* and *agg2-1*) and transgenic RNA interference Arabidopsis plants silencing *AGG1* or *AGG2* were used to reveal AGG functions in Arabidopsis (Trusov et al., 2007). Phenotypic analyses of mutants lacking either one or two Gγ subunits suggested that βγ1 works independently of βγ2 in resistance to nectrotrophic fungi, βγ1 and βγ2 work redundantly in auxin-induction of lateral root formation, and βγ1 and βγ2 work complementarily in D-glucose inhibition of seed germination (Trusov et al., 2007). These results indicate that both γ1 and γ2 can functionally associate with β1 in Arabidopsis, however, they participate in different Gβ signaling pathways (Trusov et al., 2007). Mutant phenotypes were not observed in either single or double Gγ mutants in ABA inhibition of seed germination, regulation of $K^+$ channels and stomatal movements (Trusov et al., 2008).

In summary, both phenotypic similarities and differences have been discovered between mutants lacking Gα and Gβ as well as mutants lacking Gβ and Gγs, indicating that G protein subunits can either work together, as in mammalian systems or function independently in Arabidopsis.

**Gα interacting proteins other than Gβ and Gγ in Arabidopsis**

In animal cells, when an extracellular ligand binds and activates a GPCR, the receptor will undergo a conformational change and then activate the G protein. The activated G protein will further interact with downstream effectors, inducing different signal transduction pathways in cells. However, in plants, considering there is only one Gα subunit available for the diverse roles of G protein signaling, it is speculated that plants may have many GPCRs or effectors. Surprisingly, only two putative GPCRs in plants (*GCR1* and *GCR2*) and five effectors, RGS1, AtPirin1, PD1, PLDα1 and THF1, have been experimentally identified in Arabidopsis. It is still largely unknown how these limited signaling components facilitate G protein participation in diverse pathways.

*GCR1* was predicted to have a seven transmembrane domain and was identified as a putative GPCR encoded by a single copy gene in Arabidopsis (Plakidou-Dymock et al., 1998). Overexpression of either GCR1 or GPA1 in tobacco cells induced incorporation of thymidine into DNA for DNA synthesis, and higher levels of phospholipase C (PLC) activity and inositol 1,4,5-trisphosphate ($InsP_3$) activity were observed (Apone et al., 2003). These data suggested that both PLC and $InsP_3$ are involved in GPA1 and GCR1 signaling in plants (Colucci et al., 2002; Apone et al., 2003). Evidence from co-immunoprecipitation and yeast split-ubiquitin assay showed that GPA1 can directly interact with GCR1 (Pandey and Assmann, 2004). In contrast to the *gpa1* mutant, *gcr1* was more sensitive to ABA and S1P regulation of stomatal movements than wild type (Pandey and Assmann, 2004). This result indicates that GCR1 inhibits GPA1 function in ABA and S1P signaling in guard cells. Besides GCR1, GCR2 was reported recently to be a GPCR since GCR2 was found to interact with GPA1 and was also predicted to have a seven transmembrane domain by dense alignment surface (DAS) and TMpred software (Liu et al., 2007). However, GCR2 was predicted to have one or zero transmembrane segment when a newer version of DAS-TMfilte was used (Gao et al., 2007). Predictions from TMHMM2.0 and SOSUI also suggest that GCR2 has no transmembrane segments (Gao et al., 2007; Johnston et al., 2007a). These conflicting results were suggested to be caused by the seven short hydrophobic regions in GCR2 (Illingworth et al., 2008). These

seven hydrophobic stretches can be predicted to be transmembrane domains in some algorithms but can not in most of the prediction algorithms because they are too short to actually span the membrane (Illingworth et al., 2008).

In animal cells, the GTPase activity of the activated Gα subunit is enhanced by Gα binding to a second type of proteins, regulators of G protein signaling (RGS). An RGS gene with a putative N terminal seven transmembrane domain and a C terminal RGS box has been identified in Arabidopsis *(AtRGS1)*. Both full length AtRGS1 and the C terminal RGS box interact with GPA1, and the RGS box has the ability to enhance GTPase activity (Chen et al., 2003; Willard and Siderovski, 2004). *Atrgs1* had longer hypocotyls under darkness compared to wild type, the same phenotype as was observed by overexpression of constitutively active *GPA1* in Arabidopsis (Chen et al., 2003). This result supports the notion that AtRGS1 activates GPA1 by accelerating its GTPase activity. It was also shown that the interaction between AtRGS1 and GPA1 was disrupted when the GTPase-accelerating activity of AtRGS1 was abolished by point mutation (E320K) (Johnston et al., 2007b).

By yeast two hybrid screening of an Arabidopsis λACT two-hybrid cDNA library (Kim et al., 1997), Kaufman and his colleagues found that AtPirin1 (renamed as PRN1, a transcription cofactor) and prephenate dehydratase (PD1) could physically interact with GPA1 (Lapik and Kaufman, 2003; Warpeha et al., 2006). *AtPirin* level was up-regulated by ABA and the null mutant *atpirin* was hypersensitive to ABA inhibition of seed germination, similarly, *gpa1* seed germination is also more sensitive to ABA inhibition compared to wild type (Lapik and Kaufman, 2003). These results suggest that AtPirin1 interacts with GPA1 in the seed germination pathway. The interaction between GPA1 and PD1 was confirmed via an *in vitro* pull-down method (Warpeha et al., 2006). It was shown that PD1 positively regulates blue light-induced phenylalanine accumulation in etiolated Arabidopsis seedlings and that activated GPA1 (GPA1-GTP) promotes PD1 activity (Warpeha et al., 2006).

Besides AtPirin1 and PD1, phospholipase Dalpha1 (PLDα1) is another effector that can directly interact with GPA1 in Arabidopsis. Both recombinant or native PLDα1 could be co-precipitated with the recombinant GPA1 (Zhao and Wang, 2004). The

interaction between PLDα1 and GPA1 can be increased by GDPβs, which locks Gα in its inactive form, and decreased by GTPγs, which keeps Gα in its active form. Further analysis found that PLDα1 activity is decreased and GTPase activity is increased when interaction occurs (Zhao and Wang, 2004). *PLDα1* knockout mutants were shown to be insensitive to ABA promotion of stomatal closure (Zhang et al., 2004). These data indicate that PLDα1 might interact with GPA1 and positively regulate GPA1 function in Arabidopsis guard cells.

THYLAKOID FORMATION1 (THF1) was suggested to be a candidate GPA1 interactor via the yeast two hybrid screening method, and further confirmed by co-precipitation methods *in vitro* and *in vivo*. FRET in living root epidermal cell also suggested interaction between THF1 and GPA1 (Huang et al., 2006). In contrast to PLDα1, interaction between THF1 and GPA1 was not affected by either GDP or GTP *in vitro* (Huang et al., 2006). Since the root growth rate of *thf1-1 gpa1-4* double mutants was similar to that of the *thf1-1* single mutant on either 1% or 6% D-glucose medium, THF1 might act downstream of G protein-coupled sugar signaling in regulating Arabidopsis root growth (Huang et al., 2006).

In total, only two putative GPCRs and five GPA1 effectors have been identified to date (**Table 1-2**). More effectors await investigation for a better understanding of signaling pathways of G proteins in Arabidopsis.

## Introduction to proteomics

### Proteomic methodologies

Compared to the genome, which remains almost the same throughout the life cycle of any given organism, the proteome, PROTEin complement to a genOME, is a complicated concept. A proteome refers to the sum of all proteins produced in a given cell, tissue or organism under a certain condition. A single genome can generate

numerous proteomes under specific conditions, for example, at different developmental stages and/or during different stress responses. The fact that the genome is relatively stable while the proteome is very dynamic (Tyers and Mann, 2003) makes studying the proteome far more challenging. Proteomics, the study of proteomes, takes on the challenge as an important post-genomic approach to describe the molecular events occurring in the cells. Mass-spectrometry (MS) based proteomics (gel-based and gel-independent) is one of the most powerful tools currently available in the proteomics field. Mass spectrometers have three main parts: 1) an ion source, which produces ion analytes from samples; 2) a mass analyzer, which measures the m/z (mass to charge ratio) of analytes; 3) a detector which detects signals from separated ions (Aebersold and Mann, 2003). There are two main techniques to ionize peptides: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). MALDI ionizes samples out of dry material while ESI ionizes samples out of solutions. In general, MALDI is coupled with gel-based analyses, while ESI is compatible with liquid chromatography (LC/MS) (Aebersold and Mann, 2003; Patterson and Aebersold, 2003).

The classic technique for gel-based assay is two-dimensional (2D) electrophoresis combined with MALDI-MS. By running 2D gels, many proteins can be simultaneously separated and visualized based on their pIs (isoelectric point) and molecular weights, and then identified by MS. Two dimensional PAGE was first developed in 1975 (Klose, 1975; O'Farrell, 1975). In the original 2D PAGE setup, proteins are separated based on their pIs in a gel matrix with a pH gradient, which is followed by SDS-PAGE as the second dimension of resolution, in which proteins are separated according to their molecular masses. The resolution and reproducibility of 2D PAGE have been significantly improved by use of immobilized pH gradient (IPG) strips (Bjellqvist et al., 1982), especially long IPG strips with narrow pH ranges. Protein spots of interest can be cut from 2D gels, in-gel trypsin-digested and identified by peptide mass fingerprinting (PMF) using MS or tandem mass spectrometry (MS/MS) (Monteoliva and Albar, 2004). Peptide masses obtained from MS or MS/MS are compared with those in a database containing theoretical peptide masses generated from the genome of an organism, allowing identification of the original protein. With advanced 2D analysis software, e.g.

PDQuest (Bio-Rad), the relative protein amount in each spot can be measured based on spot intensity. However, the reproducibility of this kind of quantification is limited because of inaccuracy in determination of spot boundary, the possibility of multiple proteins residing in one spot, and the possibility that one protein is present as multiple spots (Resch et al., 2006). To circumvent gel-to-gel variations and improve the reproducibility of 2D gels, differential in-gel electrophoresis (also called difference gel electrophoresis, or DIGE) was developed (Unlu et al., 1997). In this methodology, samples are labeled with fluorescent Cy Dyes (Cy2, Cy3 or Cy5) before they are mixed together and resolved on the same 2D gel. The elimination of gel matrix differences between samples leads to increased reproducibility.

Alternative and complementary technologies to circumvent the limitations of 2D gels, including poor resolving power for membrane proteins and low abundance proteins, are gel-free methods (Roe and Griffin, 2006). These shotgun methods provide an alternative to gel-dependent methods. No gels are necessary, instead, proteins are first digested with protease and then the peptides are resolved by LC/MS. Compared to the gel-based methods, these methods are faster in sample preparation but requires more time for final data analysis (Wu and Yates, 2003). Multidimensional Protein Identification Technology (MudPIT), conceptually similar to 2D PAGE as a two dimensional separation system, is a rapid and large scale proteomic analysis method. Using this technology, samples are separated sequentially on two columns: the first is a strong cation exchange column, and the second is a reversed-phase high performance liquid chromatography (HPLC) column. Peptides are identified by a tandem mass spectrometer (MS/MS) after elution from the second column. In the first MS, the mass/charge ratios of all the peptides are obtained, and in the second MS, the peptide with the most intense signal is further fragmented and sequenced (Washburn et al., 2001; Wolters et al., 2001). This technology was first introduced by Wolters et al. (2001), and has high reproducibility and greater dynamic range in peptide identification compared to the gel-based methods (Wolters et al., 2001). Using this method, the number of MS peptides detected for each protein can be used to indicate relative protein abundance (Andersen et al., 2003). Compared to the gel-free labeling methods discussed in the next paragraph,

this method is relatively easier but less accurate for quantitative proteomic studies (Cox and Mann, 2007).

The isotope-coded affinity tag (ICAT) method developed by Aebersold and co-workers was the first reported labeling method for quantitative identification of protein changes between two samples (Gygi et al., 1999). There are three elements in the ICAT reagent tag: an affinity group for purification of the ICAT-labeled peptides; a reactive group for interaction with cysteine residues in peptides, and a linker group for incorporation of heavy or light isotopes (Gygi et al., 1999). In a typical ICAT experiment, the two samples to be compared are reduced first, then each sample is labeled with a "light" or "heavy" ICAT reagent. The two samples are then pooled together for trypsin digestion, and the resulting peptides are finally purified via avidin affinity chromatography to enrich all the Cys containing peptides. Upon fragmentation by MS/MS, the relative signal intensity of the "heavy" and "light" peaks of the same peptide is correlated to the relative quantity of that peptide, thus the protein from which it is derived, in that particular sample (Schneider and Hall, 2005). Since cysteine-containing peptides are the only targets of the ICAT tag, one drawback of this method is under-estimation of proteins that have no or few free Cys residues. The Isotope Tags for Relative and Absolute Quantification (iTRAQ) reagent from ABI (Applied Biosystems), which labels lysine residues and the N termini of peptides, can be used to simultaneously and quantitatively compare up to four samples (the new release can compare up to 8 samples). Similar to ICAT, each iTRAQ tag contains three groups, a peptide reaction group, a balance group and a reporter group, and has exactly the same mass. The working principle of iTRAQ is similar to ICAT except that the step for purification of Cys-containing peptides is eliminated because all peptides can be labeled by iTRAQ reagents. In summary, compared to ICAT, ITRAQ has two advantages: 1) ITRAQ can compare up to eight samples simultaneously while ICAT can only compare two samples simultaneously; 2) ITRAQ can label all the digested peptides however ICAT can only label peptides containing cysteine residues.

**Application of proteomics to plants**

Proteomics is not only a powerful technology to identify new proteins and quantify protein abundance, but also a dissecting tool to unravel protein functions and how these functions are related to a signaling network. In fact, proteomics is becoming more and more important in deciphering protein functions. With the completion of the Arabidopsis genome sequence in 2000 (The Arabidopsis Genome Initiative, 2000), it is now feasible to apply large-scale proteomic analyses to Arabidopsis (Kersten et al., 2002). Despite their relatively advanced counterparts in animals and yeast, proteomics studies in plants are still in their infancy. For example, a PubMed search for proteomics yielded more than 13,000 papers in total since 2000, however a search for proteomics & plants yielded only about 730 papers. A search with proteomics & Arabidopsis yielded only about 270 papers during the same period. These figures may imply that the plant proteomics studies lag years behind proteomic studies in other organisms. There are two possible scientific reasons for this: first, compared to animals, plant tissues have lower protein concentration per fresh weight; second, protein extraction is difficult in plants because compounds derived from secondary metabolism can greatly affect protein extraction in plants (Giavalisco et al., 2003). One general strategy for plant proteomic sample preparation is trichloroacetic acid (TCA)/acetone precipitation followed with extraction buffers. The precipitation step both concentrates proteins and eliminates contaminating metabolites (Rose et al., 2004).

Proteomics has been applied to plants in several aspects : 1) proteomic profiling of specific organs, tissues, cells, or organelles, e.g. cell wall (Chivasa et al., 2002; Slabas et al., 2004), plasma membrane (Santoni et al., 1998), chloroplast (Ferro et al., 2002; Ferro et al., 2003), mitochondria (Kruft et al., 2001) and pollen (Holmes-Davis et al., 2005; Noir et al., 2005) (**Table 1-3**); 2) proteomic comparison, e. g. plasma membrane or nuclear proteins with or without cold treatment (Bae et al., 2003; Kawamura and Uemura, 2003), seed proteomes from WS and *ga1* (GA biosynthesis mutant) (Gallardo et al., 2002); 3) characterization of protein complexes containing a specific protein of interest,

such as the SERK1 protein interacting complex (Karlova et al., 2006) and ClpP/R/S protein complexes (Rudella et al., 2006).

DNA can be amplified by PCR technology; however, no protein amplification technique is available to date, and therefore sensitivity is a serious issue in proteomic studies. The dynamic range of protein concentrations in a cell varies up to $10^{12}$ fold (Schneider and Hall, 2005). Not surprisingly, only a few abundant "housekeeping" proteins fall in the identifiable range in the absence of pre-fractionation. To improve the detection coverage, proteins can be pre-fractionated before loading into gels or LC columns. Another limitation is that less than 10% of abundant peptides can actually be identified in a single MS analysis because of the sample complexity (Kuster et al., 2005). Despite these challenges, proteomics is still a powerful approach to help understand functional mechanisms in complex biological systems in the post-genome era.

**Overview of thesis chapters**

Despite the importance of guard cells in scientific research and agricultural applications, no proteomic study of guard cells has been reported. To globally view proteins expressed in guard cells, three complementary approaches including narrow range pH, broad range pH, and MudPIT methods were applied to profile the Arabidopsis WS guard cell proteome (Chapter 2). One protein, myrosinase 1 (TGG1), was revealed to be the most abundant guard cell protein by all three proteomic methods. The function of TGG1 in Arabidopsis guard cells was evaluated via phenotypic analysis of mutants lacking this protein (Chapter 2). Meanwhile, bioinformatic analysis suggested that proteins involved in glycolysis are enriched in guard cells. Therefore, Arabidopsis mutants with a defect in the glycolysis pathway were also studied (Chapter 2).

GPA1 is implicated in multiple biological processes in Arabidopsis. To better understand GPA1 function in guard cells, the comparative proteomic method, iTRAQ, was applied to study ABA-regulated GPA1 signaling in guard cells (Chapter 3). Leaf proteins were first used to evaluate the reproducibility and usefulness of iTRAQ in

studying Arabidopsis proteins. Guard cell proteins from Col and *gpa1-4* with presence or absence of ABA treatment were labeled with iTRAQ reagents and compared via iTRAQ technology (Chapter 3).

To study the correlation of the abundance of mRNAs and proteins in Arabidopsis guard cells, a microarray experiment was performed using WS guard cell protoplasts to obtain the transcriptome of Arabidopsis guard cells. Protein levels of the identified guard cell proteins (Chapter 2) were compared to their corresponding mRNA levels and the correlation between guard cell mRNA and protein levels was assessed (Chapter 4). To study the co-responses of mRNA and protein in response to ABA treatment or *GPA1* mutation in Arabidopsis guard cells, microarray data of Col and *gpa1-4* guard cell epidermal peels with or without ABA treatment were extracted to compare to the protein data obtained via iTRAQ. (The microarray data were obtained from a group project, and I was in charge of epidermal peel preparations for all the microarray experiments in this big project) (Chapter 4).

To better understand GPA1 functions in Arabidopsis, four different approaches were also pursued to identify GPA1 interacting proteins (Chapter 5). Finally, in Chapter 6, I propose future work based on the experimental results from Chapter 2-5.

**References**

**Adjobo-Hermans, M.J., Goedhart, J., and Gadella, T.W., Jr.** (2006). Plant G protein heterotrimers require dual lipidation motifs of Gα and Gγ and do not dissociate upon activation. J Cell Sci **119,** 5087-5097.

**Aebersold, R., and Mann, M.** (2003). Mass spectrometry-based proteomics. Nature **422,** 198-207.

**Andersen, J.S., Wilkinson, C.J., Mayor, T., Mortensen, P., Nigg, E.A., and Mann, M.** (2003). Proteomic characterization of the human centrosome by protein correlation profiling. Nature **426,** 570-574.

**Apone, F., Alyeshmerni, N., Wiens, K., Chalmers, D., Chrispeels, M.J., and Colucci, G.** (2003). The G-protein-coupled receptor GCR1 regulates DNA synthesis through activation of phosphatidylinositol-specific phospholipase C. Plant Physiol **133,** 571-579.

**Assmann, S.M.** (2002). Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell **14 Suppl,** S355-373.

**Assmann, S.M.** (2004). Plant G proteins, phytohormones, and plasticity: three questions and a speculation. Sci STKE **2004,** re20.

**Assmann, S.M.** (2005). G proteins go green: a plant G protein signaling FAQ sheet. Science **310,** 71-73.

**Bae, M.S., Cho, E.J., Choi, E.Y., and Park, O.K.** (2003). Analysis of the Arabidopsis nuclear proteome and its response to cold stress. Plant J **36,** 652-663.

**Baerenfaller, K., Grossmann, J., Grobei, M.A., Hull, R., Hirsch-Hoffmann, M., Yalovsky, S., Zimmermann, P., Grossniklaus, U., Gruissem, W., and Baginsky, S.** (2008). Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science **320,** 938-941.

**Bayer, E.M., Bottrill, A.R., Walshaw, J., Vigouroux, M., Naldrett, M.J., Thomas, C.L., and Maule, A.J.** (2006). Arabidopsis cell wall proteome defined using multidimensional protein identification technology. Proteomics **6,** 301-311.

**Bjellqvist, B., Ek, K., Righetti, P.G., Gianazza, E., Gorg, A., Westermeier, R., and Postel, W.** (1982). Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods **6,** 317-339.

**Blatt, M.R.** (1990). Potassium channel currents in intact stomatal guard cells: rapid enhancement by abscisic acid. Planta **180,** 445-455.

**Booker, F.L., Burkey, K.O., Overmyer, K., and Jones, A.M.** (2004). Differential responses of G-protein *Arabidopsis thaliana* mutants to ozone. New Phytologist **162,** 633-641.

**Boudart, G., Jamet, E., Rossignol, M., Lafitte, C., Borderies, G., Jauneau, A., Esquerre-Tugaye, M.T., and Pont-Lezica, R.** (2005). Cell wall proteins in apoplastic fluids of *Arabidopsis thaliana* rosettes: identification by mass spectrometry and bioinformatics. Proteomics **5,** 212-221.

**Briggs, W.R., Beck, C.F., Cashmore, A.R., Christie, J.M., Hughes, J., Jarillo, J.A., Kagawa, T., Kanegae, H., Liscum, E., Nagatani, A., Okada, K., Salomon, M., Rudiger, W., Sakai, T., Takano, M., Wada, M., and Watson, J.C.** (2001). The phototropin family of photoreceptors. Plant Cell **13,** 993-997.

**Carroll, A.J., Heazlewood, J.L., Ito, J., and Millar, A.H.** (2008). Analysis of the Arabidopsis cytosolic ribosome proteome provides detailed insights into its components and their post-translational modification. Mol Cell Proteomics **7,** 347-369.

**Carter, C., Pan, S., Zouhar, J., Avila, E.L., Girke, T., and Raikhel, N.V.** (2004). The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell **16,** 3285-3303.

**Casson, S., and Gray, J.E.** (2008). Influence of environmental factors on stomatal development. New Phytologist **178,** 9-23.

**Chakravorty, D., and Botella, J.R.** (2007). Over-expression of a truncated *Arabidopsis thaliana* heterotrimeric G protein γ subunit results in a phenotype similar to α and β subunit knockouts. Gene **393,** 163-170.

**Chen, J.G., Gao, Y., and Jones, A.M.** (2006a). Differential roles of Arabidopsis heterotrimeric G-protein subunits in modulating cell division in roots. Plant Physiol **141,** 887-897.

**Chen, J.G., Willard, F.S., Huang, J., Liang, J., Chasse, S.A., Jones, A.M., and Siderovski, D.P.** (2003). A seven-transmembrane RGS protein that modulates plant cell proliferation. Science **301,** 1728-1731.

**Chen, Y., Ji, F., Xie, H., Liang, J., and Zhang, J.** (2006b). The regulator of G-protein signaling proteins involved in sugar and abscisic acid signaling in Arabidopsis seed germination. Plant Physiol **140,** 302-310.

**Chivasa, S., Ndimba, B.K., Simon, W.J., Robertson, D., Yu, X.L., Knox, J.P., Bolwell, P., and Slabas, A.R.** (2002). Proteomic analysis of the *Arabidopsis thaliana* cell wall. Electrophoresis **23,** 1754-1765.

**Christie, J.M.** (2007). Phototropin blue-light receptors. Annu Rev Plant Biol **58,** 21-45.

**Colucci, G., Apone, F., Alyeshmerni, N., Chalmers, D., and Chrispeels, M.J.** (2002). GCR1, the putative Arabidopsis G protein-coupled receptor gene is cell cycle-regulated, and its overexpression abolishes seed dormancy and shortens time to flowering. Proc Natl Acad Sci U S A **99,** 4736-4741.

**Coursol, S., Fan, L.M., Le Stunff, H., Spiegel, S., Gilroy, S., and Assmann, S.M.** (2003). Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. Nature **423,** 651-654.

**Cox, J., and Mann, M.** (2007). Is proteomics the new genomics? Cell **130,** 395-398.

**Evanko, D.S., Thiyagarajan, M.M., Siderovski, D.P., and Wedegaertner, P.B.** (2001). Gβγ isoforms selectively rescue plasma membrane localization and palmitoylation of mutant $G\alpha_s$ and $G\alpha_q$. J Biol Chem **276,** 23945-23953.

**Fan, L.M., Zhao, Z., and Assmann, S.M.** (2004). Guard cells: a dynamic signaling model. Curr Opin Plant Biol **7,** 537-546.

**Fan, L.M., Zhang, W., Chen, J.G., Taylor, J.P., Jones, A.M., and Assmann, S.M.** (2008). Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ and RGS–deficient Arabidopsis lines. Proc Natl Acad Sci U S A **105,** 8476-8481.

**Ferro, M., Salvi, D., Riviere-Rolland, H., Vermat, T., Seigneurin-Berny, D., Grunwald, D., Garin, J., Joyard, J., and Rolland, N.** (2002). Integral membrane proteins of the chloroplast envelope: identification and subcellular localization of new transporters. Proc Natl Acad Sci U S A **99,** 11487-11492.

**Ferro, M., Salvi, D., Brugiere, S., Miras, S., Kowalski, S., Louwagie, M., Garin, J., Joyard, J., and Rolland, N.** (2003). Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. Mol Cell Proteomics **2,** 325-345.

**Fishburn, C.S., Pollitt, S.K., and Bourne, H.R.** (2000). Localization of a peripheral membrane protein: Gβγ targets $G\alpha_Z$. Proc Natl Acad Sci U S A **97,** 1085-1090.

**Gallardo, K., Job, C., Groot, S.P., Puype, M., Demol, H., Vandekerckhove, J., and Job, D.** (2002). Proteomics of Arabidopsis seed germination. A comparative study of wild-type and gibberellin-deficient seeds. Plant Physiol **129,** 823-837.

**Gao, Y., Zeng, Q., Guo, J., Cheng, J., Ellis, B.E., and Chen, J.G.** (2007). Genetic characterization reveals no role for the reported ABA receptor, GCR2, in ABA control of seed germination and early seedling development in Arabidopsis. Plant J **52,** 1001-1013.

**Ghelis, T., Dellis, O., Jeannette, E., Bardat, F., Miginiac, E., and Sotta, B.** (2000). Abscisic acid plasmalemma perception triggers a calcium influx essential for RAB18 gene expression in *Arabidopsis thaliana* suspension cells. FEBS Lett **483,** 67-70.

**Giavalisco, P., Nordhoff, E., Lehrach, H., Gobom, J., and Klose, J.** (2003). Extraction of proteins from plant tissues for two-dimensional electrophoresis analysis. electrophoresis **24,** 207-216.

**Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., and Aebersold, R.** (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol **17,** 994-999.

**Holmes-Davis, R., Tanaka, C.K., Vensel, W.H., Hurkman, W.J., and McCormick, S.** (2005). Proteome mapping of mature pollen of *Arabidopsis thaliana*. Proteomics **5,** 4864-4884.

**Hooks, S.B., Martemyanov, K., and Zachariou, V.** (2008). A role of RGS proteins in drug addiction. Biochem Pharmacol **75,** 76-84.

**Huang, J., Taylor, J.P., Chen, J.G., Uhrig, J.F., Schnell, D.J., Nakagawa, T., Korth, K.L., and Jones, A.M.** (2006). The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in Arabidopsis. Plant Cell **18,** 1226-1238.

**Hugouvieux, V., Kwak, J.M., and Schroeder, J.I.** (2001). An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis. Cell **106,** 477-487.

**Ichida, A.M., Pei, Z.M., Baizabal-Aguirre, V.M., Turner, K.J., and Schroeder, J.I.** (1997). Expression of a $Cs^+$-resistant guard cell $K^+$ channel confers $Cs^+$-resistant, light-induced stomatal opening in transgenic Arabidopsis. Plant Cell **9,** 1843-1857.

**Illingworth, C.J., Parkes, K.E., Snell, C.R., Mullineaux, P.M., and Reynolds, C.A.** (2008). Criteria for confirming sequence periodicity identified by Fourier

transform analysis: application to GCR2, a candidate plant GPCR? Biophys Chem **133,** 28-35.

**Israelsson, M., Siegel, R.S., Young, J., Hashimoto, M., Iba, K., and Schroeder, J.I.** (2006). Guard cell ABA and $CO_2$ signaling network updates and $Ca^{2+}$ sensor priming hypothesis. Curr Opin Plant Biol **9,** 654-663.

**Johnston, C.A., Temple, B.R., Chen, J.G., Gao, Y., Moriyama, E.N., Jones, A.M., Siderovski, D.P., and Willard, F.S.** (2007a). Comment on "A G protein coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid". Science **318,** 914; author reply 914.

**Johnston, C.A., Taylor, J.P., Gao, Y., Kimple, A.J., Grigston, J.C., Chen, J.G., Siderovski, D.P., Jones, A.M., and Willard, F.S.** (2007b). GTPase acceleration as the rate-limiting step in Arabidopsis G protein-coupled sugar signaling. Proc Natl Acad Sci U S A **104,** 17317-17322.

**Jones, A.M., Ecker, J.R., and Chen, J.G.** (2003). A reevaluation of the role of the heterotrimeric G protein in coupling light responses in Arabidopsis. Plant Physiol **131,** 1623-1627.

**Joo, J.H., Wang, S., Chen, J.G., Jones, A.M., and Fedoroff, N.V.** (2005). Different signaling and cell death roles of heterotrimeric G protein α and β subunits in the Arabidopsis oxidative stress response to ozone. Plant Cell **17,** 957-970.

**Karlova, R., Boeren, S., Russinova, E., Aker, J., Vervoort, J., and de Vries, S.** (2006). The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE1. Plant Cell **18,** 626-638.

**Kawamura, Y., and Uemura, M.** (2003). Mass spectrometric approach for identifying putative plasma membrane proteins of Arabidopsis leaves associated with cold acclimation. Plant J **36,** 141-154.

**Kersten, B., Burkle, L., Kuhn, E.J., Giavalisco, P., Konthur, Z., Lueking, A., Walter, G., Eickhoff, H., and Schneider, U.** (2002). Large-scale plant proteomics. Plant Mol Biol **48,** 133-141.

**Kim, J., Harter, K., and Theologis, A.** (1997). Protein-protein interactions among the Aux/IAA proteins. Proc Natl Acad Sci U S A **94,** 11786-11791.

**Kinoshita, T., and Shimazaki, K.** (1999). Blue light activates the plasma membrane $H^+$-ATPase by phosphorylation of the C-terminus in stomatal guard cells. Embo J **18,** 5548-5558.

**Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M., and Shimazaki, K.** (2001). Phot1 and phot2 mediate blue light regulation of stomatal opening. Nature **414,** 656-660.

**Kleffmann, T., Russenberger, D., von Zychlinski, A., Christopher, W., Sjolander, K., Gruissem, W., and Baginsky, S.** (2004). The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biol **14,** 354-362.

**Klose, J.** (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Human genetics **26,** 231-243.

**Kruft, V., Eubel, H., Jansch, L., Werhahn, W., and Braun, H.P.** (2001). Proteomic approach to identify novel mitochondrial proteins in Arabidopsis. Plant Physiol **127,** 1694-1710.

**Kuster, B., Schirle, M., Mallick, P., and Aebersold, R.** (2005). Scoring proteomes with proteotypic peptide probes. Nat Rev Mol Cell Biol **6,** 577-583.

**Kwak, J.M., Moon, J.H., Murata, Y., Kuchitsu, K., Leonhardt, N., DeLong, A., and Schroeder, J.I.** (2002). Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, RCN1, confers abscisic acid insensitivity in Arabidopsis. Plant Cell **14,** 2849-2861.

**Kwak, J.M., Murata, Y., Baizabal-Aguirre, V.M., Merrill, J., Wang, M., Kemper, A., Hawke, S.D., Tallman, G., and Schroeder, J.I.** (2001). Dominant negative guard cell $K^+$ channel mutants reduce inward-rectifying $K^+$ currents and light-induced stomatal opening in Arabidopsis. Plant Physiol **127,** 473-485.

**Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., and Schroeder, J.I.** (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis. Embo J **22,** 2623-2633.

**Lapik, Y.R., and Kaufman, L.S.** (2003). The Arabidopsis cupin domain protein AtPirin1 interacts with the G protein alpha-subunit GPA1 and regulates seed germination and early seedling development. Plant Cell **15,** 1578-1590.

**Lasceve, G., Leymarie, J., and Vavasseur, A.** (1997). Alterations in light-induced stomatal opening in a starch-deficient mutant of *Arabidopsis thaliana* L deficient in chloroplast phosphoglucomutase activity. Plant Cell and Environment **20,** 350-358.

**Lease, K.A., Wen, J., Li, J., Doke, J.T., Liscum, E., and Walker, J.C.** (2001). A mutant Arabidopsis heterotrimeric G-protein β subunit affects leaf, flower, and fruit development. Plant Cell **13,** 2631-2641.

**Lee, J., Garrett, W.M., and Cooper, B.** (2007). Shotgun proteomic analysis of *Arabidopsis thaliana* leaves. J Sep Sci **30,** 2225-2230.

**Lemichez, E., Wu, Y., Sanchez, J.-P., Mettouchi, A., Mathur, J., and Chua, N.-H.** (2001). Inactivation of AtRac1 by abscisic acid is essential for stomatal closure. Genes & Dev. **15,** 1808-1816.

**Li, S., Assmann, S.M., and Albert, R.** (2006). Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. PLoS Biol **4,** e312.

**Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W.H., and Ma, L.** (2007). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science **315,** 1712-1716.

**Llorente, F., Alonso-Blanco, C., Sanchez-Rodriguez, C., Jorda, L., and Molina, A.** (2005). ERECTA receptor-like kinase and heterotrimeric G protein from Arabidopsis are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant J **43,** 165-180.

**Ma, H., Yanofsky, M.F., and Meyerowitz, E.M.** (1990). Molecular cloning and characterization of GPA1, a G protein α subunit gene from *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **87,** 3821-3825.

**Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C.** (1997). FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell **89,** 737-745.

**MacRobbie, E.A.** (1998). Signal transduction and ion channels in guard cells. Philos Trans R Soc Lond B Biol Sci **353,** 1475-1488.

**Marmagne, A., Rouet, M.A., Ferro, M., Rolland, N., Alcon, C., Joyard, J., Garin, J., Barbier-Brygoo, H., and Ephritikhine, G.** (2004). Identification of new intrinsic proteins in Arabidopsis plasma membrane proteome. Mol Cell Proteomics **3,** 675-691.

**Mason, M.G., and Botella, J.R.** (2000). Completing the heterotrimer: isolation and characterization of an *Arabidopsis thaliana* G protein γ-subunit cDNA. Proc Natl Acad Sci U S A **97,** 14784-14788.

**Mason, M.G., and Botella, J.R.** (2001). Isolation of a novel G-protein γ-subunit from *Arabidopsis thaliana* and its interaction with Gβ. Biochim Biophys Acta **1520,** 147-153.

**McCudden, C.R., Hains, M.D., Kimple, R.J., Siderovski, D.P., and Willard, F.S.** (2005). G-protein signaling: back to the future. Cell Mol Life Sci **62,** 551-577.

**Merlot, S., Leonhardt, N., Fenzi, F., Valon, C., Costa, M., Piette, L., Vavasseur, A., Genty, B., Boivin, K., Muller, A., Giraudat, J., and Leung, J.** (2007). Constitutive activation of a plasma membrane $H^+$-ATPase prevents abscisic acid-mediated stomatal closure. Embo J **26,** 3216-3226.

**Mishra, G., Zhang, W., Deng, F., Zhao, J., and Wang, X.** (2006). A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science **312,** 264-266.

**Mochizuki, N., Brusslan, J.A., Larkin, R., Nagatani, A., and Chory, J.** (2001). Arabidopsis genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc Natl Acad Sci U S A **98,** 2053-2058.

**Monteoliva, L., and Albar, J.P.** (2004). Differential proteomics: an overview of gel and non-gel based approaches. Brief Funct Genomic Proteomic **3,** 220-239.

**Mori, I.C., Murata, Y., Yang, Y., Munemasa, S., Wang, Y.F., Andreoli, S., Tiriac, H., Alonso, J.M., Harper, J.F., Ecker, J.R., Kwak, J.M., and Schroeder, J.I.** (2006). CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and $Ca^{2+}$-permeable channels and stomatal closure. PLoS Biol **4,** e327.

**Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J.** (2002). Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell **14,** 3089-3099.

**Ng, C.K., Carr, K., McAinsh, M.R., Powell, B., and Hetherington, A.M.** (2001). Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. Nature **410,** 596-599.

**Noir, S., Brautigam, A., Colby, T., Schmidt, J., and Panstruga, R.** (2005). A reference map of the *Arabidopsis thaliana* mature pollen proteome. Biochem Biophys Res Commun **337,** 1257-1266.

**O'Farrell, P.H.** (1975). High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250,** 4007-4021.

**Osakabe, Y., Maruyama, K., Seki, M., Satou, M., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2005). Leucine-rich repeat receptor-like kinase1 is a key membrane-bound regulator of abscisic acid early signaling in Arabidopsis. Plant Cell **17,** 1105-1119.

**Pandey, S., and Assmann, S.M.** (2004). The Arabidopsis putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. Plant Cell **16,** 1616-1632.

**Pandey, S., Zhang, W., and Assmann, S.M.** (2007). Roles of ion channels and transporters in guard cell signal transduction. FEBS Lett **581,** 2325-2336.

**Pandey, S., Chen, J.G., Jones, A.M., and Assmann, S.M.** (2006). G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. Plant Physiol **141,** 243-256.

**Patterson, S.D., and Aebersold, R.H.** (2003). Proteomics: the first decade and beyond. Nat Genet **33 Suppl,** 311-323.

**Pei, Z.M., Ghassemian, M., Kwak, C.M., McCourt, P., and Schroeder, J.I.** (1998). Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science **282,** 287-290.

**Perfus-Barbeoch, L., Jones, A.M., and Assmann, S.M.** (2004). Plant heterotrimeric G protein function: insights from Arabidopsis and rice mutants. Curr Opin Plant Biol **7,** 719-731.

**Plakidou-Dymock, S., Dymock, D., and Hooley, R.** (1998). A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins. Curr Biol **8,** 315-324.

**Pyne, S., and Pyne, N.J.** (2000). Sphingosine 1-phosphate signalling in mammalian cells. Biochem J **349,** 385-402.

**Razem, F.A., El-Kereamy, A., Abrams, S.R., and Hill, R.D.** (2006). The RNA-binding protein FCA is an abscisic acid receptor. Nature **439,** 290-294.

**Razem, F.A., Luo, M., Liu, J.H., Abrams, S.R., and Hill, R.D.** (2004). Purification and characterization of a barley aleurone abscisic acid-binding protein. J Biol Chem **279,** 9922-9929.

**Resch, A., Leicht, S., Saric, M., Pasztor, L., Jakob, A., Gotz, F., and Nordheim, A.** (2006). Comparative proteome analysis of Staphylococcus aureus biofilm and planktonic cells and correlation with transcriptome profiling. Proteomics **6,** 1867-1877.

**Roe, M.R., and Griffin, T.J.** (2006). Gel-free mass spectrometry-based high throughput proteomics: tools for studying biological response of proteins and proteomes. Proteomics **6,** 4678-4687.

**Rose, J.K., Bashir, S., Giovannoni, J.J., Jahn, M.M., and Saravanan, R.S.** (2004). Tackling the plant proteome: practical approaches, hurdles and experimental tools. Plant J **39,** 715-733.

**Rudella, A., Friso, G., Alonso, J.M., Ecker, J.R., and van Wijk, K.J.** (2006). Downregulation of ClpR2 leads to reduced accumulation of the ClpPRS protease

complex and defects in chloroplast biogenesis in Arabidopsis. Plant Cell **18,** 1704-1721.

**Saez, A., Robert, N., Maktabi, M.H., Schroeder, J.I., Serrano, R., and Rodriguez, P.L.** (2006). Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. Plant Physiol **141,** 1389-1399.

**Santoni, V., Rouquie, D., Doumas, P., Mansion, M., Boutry, M., Degand, H., Dupree, P., Packman, L., Sherrier, J., Prime, T., Bauw, G., Posada, E., Rouze, P., Dehais, P., Sahnoun, I., Barlier, I., and Rossignol, M.** (1998). Use of a proteome strategy for tagging proteins present at the plasma membrane. Plant J **16,** 633-641.

**Schneider, L.V., and Hall, M.P.** (2005). Stable isotope methods for high-precision proteomics. Drug Discov Today **10,** 353-363.

**Schroeder, J.I., Raschke, K., and Neher, E.** (1987). Voltage dependence of $K^+$ channels in guard-cell protoplasts. Proc Natl Acad Sci U S A **84,** 4108-4112.

**Schroeder, J.I., Kwak, J.M., and Allen, G.J.** (2001a). Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature **410,** 327-330.

**Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., and Waner, D.** (2001b). Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol **52,** 627-658.

**Schwartz, A., Wu, W.H., Tucker, E.B., and Assmann, S.M.** (1994). Inhibition of inward $K^+$ channels and stomatal response by abscisic acid: an intracellular locus of phytohormone action. Proc Natl Acad Sci U S A **91,** 4019-4023.

**Seo, M., and Koshiba, T.** (2002). Complex regulation of ABA biosynthesis in plants. Trends Plant Sci **7,** 41-48.

**Shen, Y.Y., Wang, X.F., Wu, F.Q., Du, S.Y., Cao, Z., Shang, Y., Wang, X.L., Peng, C.C., Yu, X.C., Zhu, S.Y., Fan, R.C., Xu, Y.H., and Zhang, D.P.** (2006). The Mg-chelatase H subunit is an abscisic acid receptor. Nature **443,** 823-826.

**Shimazaki, K., Doi, M., Assmann, S.M., and Kinoshita, T.** (2007). Light regulation of stomatal movement. Annu Rev Plant Biol **58,** 219-247.

**Slabas, A.R., Ndimba, B., Simon, W.J., and Chivasa, S.** (2004). Proteomic analysis of the Arabidopsis cell wall reveals unexpected proteins with new cellular locations. Biochem Soc Trans **32,** 524-528.

**Temple, B.R., and Jones, A.M.** (2007). The plant heterotrimeric G-protein complex. Annu Rev Plant Biol **58,** 249-266.

**The Arabidopsis Genome Initiative, a.** (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408,** 796-815.

**Trusov, Y., Zhang, W., Assmann, S.M., and Botella, J.R.** (2008). $G\gamma1 + G\gamma2 \neq G\beta$: heterotrimeric G protein Gγ-deficient mutants do not recapitulate all phenotypes of Gβ-deficient mutants. Plant Physiol **147,** 636-649.

**Trusov, Y., Rookes, J.E., Chakravorty, D., Armour, D., Schenk, P.M., and Botella, J.R.** (2006). Heterotrimeric G proteins facilitate Arabidopsis resistance to necrotrophic pathogens and are involved in jasmonate signaling. Plant Physiol **140,** 210-220.

**Trusov, Y., Rookes, J.E., Tilbrook, K., Chakravorty, D., Mason, M.G., Anderson, D., Chen, J.G., Jones, A.M., and Botella, J.R.** (2007). Heterotrimeric G protein γ subunits provide functional selectivity in Gβγ dimer signaling in Arabidopsis. Plant Cell **19,** 1235-1250.

**Tyers, M., and Mann, M.** (2003). From genomics to proteomics. Nature **422,** 193-197.

**Ullah, H., Chen, J.G., Wang, S., and Jones, A.M.** (2002). Role of a heterotrimeric G protein in regulation of Arabidopsis seed germination. Plant Physiol **129,** 897-907.

**Ullah, H., Chen, J.G., Young, J.C., Im, K.H., Sussman, M.R., and Jones, A.M.** (2001). Modulation of cell proliferation by heterotrimeric G protein in Arabidopsis. Science **292,** 2066-2069.

**Ullah, H., Chen, J.G., Temple, B., Boyes, D.C., Alonso, J.M., Davis, K.R., Ecker, J.R., and Jones, A.M.** (2003). The β-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell **15,** 393-409.

**Unlu, M., Morgan, M.E., and Minden, J.S.** (1997). Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis **18,** 2071-2077.

**Wang, S., Narendra, S., and Fedoroff, N.** (2007). Heterotrimeric G protein signaling in the Arabidopsis unfolded protein response. Proc Natl Acad Sci U S A **104,** 3817-3822.

**Wang, S., Assmann, S.M., and Fedoroff, N.V.** (2008). Characterization of the Arabidopsis heterotrimeric G protein. J Biol Chem. **283**, 13913-13922.

**Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M.** (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science **292,** 2070-2072.

**Warpeha, K.M., Lateef, S.S., Lapik, Y., Anderson, M., Lee, B.-S., and Kaufman, L.S.** (2006). G-protein-coupled receptor 1, G-protein Gα-subunit 1, and prephenate dehydratase 1 are required for blue light-induced production of phenylalanine in etiolated Arabidopsis. Plant Physiol. **140,** 844-855.

**Washburn, M.P., Wolters, D., and Yates, J.R., 3rd.** (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol **19,** 242-247.

**Weiss, C.A., Garnaat, C.W., Mukai, K., Hu, Y., and Ma, H.** (1994). Isolation of cDNAs encoding guanine nucleotide-binding protein β-subunit homologues from maize (ZGB1) and Arabidopsis (AGB1). Proc Natl Acad Sci U S A **91,** 9554-9558.

**Wienkoop, S., Zoeller, D., Ebert, B., Simon-Rosin, U., Fisahn, J., Glinski, M., and Weckwerth, W.** (2004). Cell-specific protein profiling in *Arabidopsis thaliana* trichomes: identification of trichome-located proteins involved in sulfur metabolism and detoxification. Phytochemistry **65,** 1641-1649.

**Willard, F.S., and Siderovski, D.P.** (2004). Purification and *in vitro* functional analysis of the *Arabidopsis thaliana* regulator of G-protein signaling-1. Methods Enzymol **389,** 320-338.

**Wolters, D.A., Washburn, M.P., and Yates, J.R., 3rd.** (2001). An automated multidimensional protein identification technology for shotgun proteomics. Anal Chem **73,** 5683-5690.

**Wu, C.C., and Yates, J.R., 3rd.** (2003). The application of mass spectrometry to membrane proteomics. Nat Biotechnol **21,** 262-267.

**Xie, Z., Ruas, P., and Shen, Q.J.** (2005). Regulatory networks of the phytohormone abscisic acid. Vitam Horm **72,** 235-269.

**Xing, Y., Jia, W., and Zhang, J.** (2007). AtMEK1 mediates stress-induced gene expression of CAT1 catalase by triggering $H_2O_2$ production in Arabidopsis. J Exp Bot **58,** 2969-2981.

**Zeng, Q., Wang, X., and Running, M.P.** (2007). Dual lipid modification of Arabidopsis Gγ-subunits is required for efficient plasma membrane targeting. Plant Physiol **143,** 1119-1131.

**Zhang, D.P., Wu, Z.Y., Li, X.Y., and Zhao, Z.X.** (2002). Purification and identification of a 42-kilodalton abscisic acid-specific-binding protein from epidermis of broad bean leaves. Plant Physiol **128,** 714-725.

**Zhang, W., Qin, C., Zhao, J., and Wang, X.** (2004). Phospholipase Dα 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci U S A **101,** 9508-9513.

**Zhang, X., Takemiya, A., Kinoshita, T., and Shimazaki, K.** (2007). Nitric oxide inhibits blue light-specific stomatal opening via abscisic acid signaling pathways in Vicia guard cells. Plant Cell Physiol **48,** 715-723.

**Zhao, J., and Wang, X.** (2004). Arabidopsis phospholipase Dα1 interacts with the heterotrimeric G-protein α-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. J Biol Chem **279,** 1794-1800.

**Zheng, Z.L., Nafisi, M., Tam, A., Li, H., Crowell, D.N., Chary, S.N., Schroeder, J.I., Shen, J., and Yang, Z.** (2002). Plasma membrane-associated ROP10 small GTPase is a specific negative regulator of abscisic acid responses in Arabidopsis. Plant Cell **14,** 2787-2797.

**Zhu, S.Y., Yu, X.C., Wang, X.J., Zhao, R., Li, Y., Fan, R.C., Shang, Y., Du, S.Y., Wang, X.F., Wu, F.Q., Xu, Y.H., Zhang, X.Y., and Zhang, D.P.** (2007). Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. Plant Cell **19,** 3019-3036.

**Table 1-1. Twenty-four proteins have been identified to be involved in ABA signaling in Arabidopsis guard cells.**

| Protein name | AGI | Mutant phnotype | Protein characterization | Reference |
|---|---|---|---|---|
| ABH1 | At2g13540 | Hypersensitive to closure | mRNA cap binding protein | (Hugouvieux et al., 2001) |
| ABI1 | At4g26080 | Hypersensitive to closure | Protein phosphatases | (Saez et al., 2006) |
| AGB1 | At4g34460 | Hyposensitive to opening and closure | Gβ subunit | (Fan et al., 2008) |
| HAB1 | At1g72770 | Hypersensitive to closure | Protein phosphatases | (Saez et al., 2006) |
| MEK1 | At4g26070 | Hyposensitive to closure | Protein kinase | (Xing et al., 2007) |
| RAC1 | At2g17800 | Dominant positive mutant hyposensitive to closure | GTPase | (Lemichez et al., 2001) |
| AtrbohD | At5g47910 | Hyposensitive to closure | NADPH oxidase | (Kwak et al., 2003) |
| AtrbohF | At1g64060 | Hyposensitive to closure | NADPH oxidase | (Kwak et al., 2003) |
| CHLH | At5g13630 | Insensitive to opening and closure | ABA receptor | (Shen et al., 2006) |
| CPK3 | At4g23650 | Hyposensitive to closure | Protein kinase | (Mori et al., 2006) |
| CPK4 | At4g09570 | Insensitive to opening and closure | Protein kinase | (Zhu et al., 2007) |
| CPK6 | At2g17290 | Hyposensitive to closure | Protein kinase | (Mori et al., 2006) |
| CPK11 | At1g35670 | Insensitive to opening and closure | Protein kinase | (Zhu et al., 2007) |
| ERA1 | At5g40280 | Hypersensitive to closure | Farnesyltransferase | (Pei et al., 1998) |
| GCR1 | At1g48270 | Hypersensitive to closure and opening | G protein coupled receptor | (Pandey and Assmann, 2004) |
| GPA1 | At2g26300 | Hyposensitive to opening | Gα subunit | (Wang et al., 2001) |
| OST1 | At4g33950 | Hyposensitive to opening and closure | Protein kinase | (Mustilli et al., 2002) |
| OST2 | At2g18960 | Hyposensitive to closure | H-ATPase | (Merlot et al., 2007) |
| PLDα1 | At3g15730 | Insensitive | Production of PA | (Mishra et al., 2006) |
| RCN1 | At1g13320 | Hyposensitive to closure | Protein phosphatases | (Kwak et al., 2002) |
| ROP10 | At3g48040 | Hypersensitive to closure | GTPase | (Zheng et al., 2002) |
| RPK1 | At1g69270 | Insensitive to closure | Protein kinase | (Osakabe et al., 2005) |
| TGG1 | At5g26000 | Insensitive to opening | Myrosinase | (Zhao et al, submitted) |
| GCR2 | At1g52920 | Insensitive to opening and closure | ABA receptor | (Liu et al., 2007) |

Opening=ABA-inhibition of stomatal opening, closure=ABA-promotion of stomatal closure.

**Table 1-2**. **Seven proteins have been identified to interact with GPA1, in addition to Gβ and Gγ subunits.**

| Protein name | AGI | Method | Reference |
|---|---|---|---|
| GCR1 | At1g48270 | Yeast split-ubiquition assay, *in vitro* pull down assay, *in vivo* co-immunoprecipitation | (Pandey and Assmann, 2004) |
| GCR2 | At1g52950 | Yeast split-ubiquition assay, *in vivo* co-immunoprecipitation, FRET | (Gao et al., 2007; Johnston et al., 2007a; Liu et al., 2007) |
| AtRGS1 | At3g26090 | Yeast split-ubiquition assay, *in vivo* and *in vitro* Co-immunoprecipitation, FRET | (Chen et al., 2003; Johnston et al., 2007b) |
| PLDα1 | At3g15730 | *in vivo* and *in vitro* Co-immunoprecipitation | (Zhao and Wang, 2004) |
| AtPirin (PRN1) | At3g59220 | Yeast two-hybrid, *in vitro* binding assay | (Lapik and Kaufman, 2003) |
| PD1 | At2g27820 | Yeast two-hybrid, *in vitro* pull down assay, | (Warpeha et al., 2006) |
| THF1 | At2g20890 | Yeast two-hybrid, *in vivo* and *in vitro* co-immunoprecipitation, FRET | (Huang et al., 2006) |

**Table 1-3**. **Numbers of proteins identified from Arabidopsis by proteomic studies.**

| Plant material | # of identified proteins | Reference |
|---|---|---|
| Cell wall | 268* | (Slabas et al., 2004; Boudart et al., 2005; Bayer et al., 2006) |
| Chloroplast | 655 | (Kleffmann et al., 2004) |
| Epidermis | 26 | (Wienkoop et al., 2004) |
| Flowers | 7040 | (Baerenfaller et al., 2008) |
| Leaf | 2887 | (Lee et al., 2007)(1) |
| Leaf | 4853 | (Baerenfaller et al., 2008)(2) |
| Plasma membrane | 120 | (Marmagne et al., 2004) |
| Pollen | 144* | (Holmes-Davis et al., 2005; Noir et al., 2005) |
| Ribosome | 242 | (Carroll et al., 2008) |
| Root | 6125 | (Baerenfaller et al., 2008) |
| Seed | 3789 | (Baerenfaller et al., 2008) |
| Silique | 5779 | (Baerenfaller et al., 2008) |
| Trichome | 61 | (Wienkoop et al., 2004) |
| Vacuole | 402 | (Carter et al., 2004) |

*These numbers are combined from multiple published papers.
Identified leaf proteins from (1) cannot be combined with those from (2) because the protein list cannot be extracted from (2).

# Chapter 2

## Functional proteomics of *Arabidopsis thaliana* guard cells uncovers new stomatal signaling pathways

In this chapter, the electrophysiological data were obtained by Dr. Wei Zhang. All other results were generated by Zhixin Zhao.

**Abstract**

We isolated a total of $3x10^8$ guard cell protoplasts from 22,000 Arabidopsis plants and identified 1,764 unique proteins using three complementary proteomic methods: protein spot identification from broad and narrow pH range 2D gels, and 2D LC-MALDI MudPIT. This is the most comprehensive single cell type proteome from a multicellular organism to date. Myrosinase 1 (TGG1), which catalyzes the production of toxic isothiocyanates from glucosinolates, showed striking abundance in the guard cell proteome. *tgg1* mutants were hyposensitive to abscisic acid (ABA)-inhibition of guard cell inward $K^+$ channels and stomatal opening, revealing that the glucosinolate-myrosinase system, previously identified only as a defense against biotic invaders, is central to abiotic stress responses. Proteins involved in energy production were enriched in the guard cell proteome, and mutants lacking the glycolytic enzyme iPGM (2,3-biphosphoglycerate-independent phosphoglycerate mutase) had defects in stomatal movements, growth, and pollen production. Our results exemplify how enhanced knowledge of the signaling networks of a specific cell type can be gained by proteomics approaches.

**Introduction**

Multicellular organisms develop specialized cell types, each with unique functions with regard to its specific role in the organism. The importance of single-cell-type transcriptomics studies in elucidating the functions of specialized cell types is uncontested (Dinneny et al., 2008). Single cell-type proteomics studies are also essential in order to unravel the functions of specialized cells, particularly for cell types, like the guard cell, where essential responses to stimuli can occur within seconds (Assmann and Grantz, 1990) and thus are unlikely to be mediated by transcriptomic changes. However, there have been very few single cell-type proteomics studies in either plant or metazoan systems to date, in part owing to the greater complexity of the proteome and the greater technical challenges of proteomic methodologies (Tyers and Mann, 2003).

The most common subjects for single cell type proteomic studies have been cultured mammalian cell lines, where material is not limiting for proteomic analyses. Proteomic studies on cancer and neuronal cell lines have provided valuable information for basic and clinical studies (Schirle et al., 2003; Diks and Peppelenbosch, 2004). By contrast, for plant cells only the proteomes of Arabidopsis and tobacco trichomes (Wienkoop et al., 2004; Amme et al., 2005), Arabidopsis epidermal cells (Wienkoop et al., 2004), and soybean root hairs (Wan et al., 2005) have been assessed and in each case only a handful of proteins were identified: 63 from Arabidopsis trichomes, 35 from tobacco trichomes, 26 from Arabidopsis epidermal cells and 36 from root hairs. Arabidopsis pollen (a tricell microspore) has been more widely studied using proteomics because of the relative ease of isolation, however, only 135 (Holmes-Davis et al., 2005) and 121 (Noir et al., 2005) proteins have been identified from these studies. Here we report the most extensive single cell type proteome to date: the identification of 1,764 proteins in the guard cell proteome.

The guard cell is a specialized cell type located in the epidermes of higher plants. Each pair of guard cells delineates a microscopic pore called a stoma. Carbon dioxide ($CO_2$) uptake, essential for photosynthesis, as well as water vapor loss, occur through stomatal pores and are controlled by guard cell movements, regulated by changes in the turgor pressure and volume of guard cells. The study of guard cell function is particularly topical given that climate change models predict that global warming will result in more frequent and more severe droughts (Breshears et al., 2005; Schroter et al., 2005), and that stomata have been implicated in “physiological forcing” of the global water cycle (Hetherington and Woodward, 2003; Betts et al., 2007).

In addition to the central importance of guard cell function to terrestrial vegetation, the guard cell system has also become a premier model system in plant cell biology. guard cells respond autonomously, directly, rapidly, and reversibly to diverse environmental cues including light, $CO_2$, oxidative stress, humidity, and pathogens (Blatt, 2000; Israelsson et al., 2006; Pandey et al., 2007; Shimazaki et al., 2007; Fan et al., 2008b). Stomatal apertures can be easily observed under the microscope and direct measurement of ion channel and pump activity can be attained by electrophysiological

assays. Ions ($Ca^{2+}$, $K^{+}$, $Cl^{-}$, $malate^{2-}$, $NO_3^{-}$), signaling elements (e.g. G-proteins, phospholipase C (PLC), phospholipase D (PLD), inositol 1,4,5-triphosphate ($InsP_3$), nitric oxide (NO), reactive oxygen species (ROS)), and plant hormones (ABA, auxin, ethylene, cytokinins). With the advent of systems biology techniques, a dynamic model for induction of stomatal closure by the drought and stress hormone, ABA, has been developed (Li et al., 2006).

However, despite these achievements, fundamental questions remain unanswered: what is the most abundant protein in guard cells, which kinds of proteins are more likely to be expressed in guard cells, how does the composition of the guard cell proteome compare with that of other plant cell types, and how can proteomics inform studies of guard cell function? These questions are addressed by the present study. We characterized the guard cell proteome and then used genetic approaches to functionally investigate key proteins identified in that proteome. We discovered that the most abundant guard cell protein, TGG1, previously known only as an enzyme in the secondary metabolism of glucosinolates, is a shared intracellular component between abiotic and biotic stress signaling pathways in guard cells, and that iPGM proteins, enzymes of primary metabolism (glycolysis), are key components for stomatal movements, vegetative, and reproductive growth. These analyses have thus uncovered novel roles for these known enzymes.

## Results

### Proteomic methods and guard cell proteins

A major challenge in single cell type proteomics is obtaining a sufficient quantity of highly pure cells. For guard cells, as for some other plant cell types (Birnbaum et al., 2003), such purity can only be achieved by isolation of protoplasts. Numerous previous studies have validated that guard cell protoplasts (GCP) retain key physiological

responses present in guard cell *in situ*, including responsiveness to environmental signals such as light, ABA, and $CO_2$. To obtain enough GCPs for our proteomic study, we adapted our preparation method (Pandey et al., 2002) by increasing the plant material from 100 to 300 leaves per isolation. Three complementary proteomics methods were used: broad pH range 2D gels (BR, **Figure 2-1C**), narrow pH range 2D gels method (NR, **Figure 2-1D**) and 2D Liquid Chromatography – Matrix Assisted Laser Desorption/Ionization MUltiDimensional Protein Identification Technology (2D LC-MALDI MudPIT).

For the gel-based methods, stained spots were in-gel digested by trypsin, identified by MALDI-tandem time-of-flight (TOF/TOF) and analyzed with Mascot software (see Material and methods). In two independent biological replicates of BR 2D gels, 138 spots were consistently detected, and from these, 85 spots, representing 58 unique proteins, were accepted as identified, meeting a very stringent acceptance criterion of requiring a confidence interval (C.I) >99.5% for identification. To identify a greater number of proteins, GCP proteins were first prefractionated into five fractions with pH ranges 3-4.6, 4.6-5.4, 5.4-6.2, 6.2-7.0, and 7.0-10.0 using an IEF fractionator. Each fraction was applied to a corresponding single pH 2D gel (see Material and methods). A total of 250 spots was consistently detected from two biological replicates, from which 120 spots were identified with C.I > 99.5%; these spots represent 59 unique proteins. By far the largest protein spot on either BR or NR 2D gels was the myrosinase TGG1 (**Figure 2-1C**, spot 12, **Figure 2-1D**, spot 3E12). Multiple TGG1 spots were detected on 2D gels (**Table 2-1**). Fourteen and 19 proteins were identified in multiple spots on the BR and NR 2D gels, respectively, representing a total of 28 unique proteins. (**Table 2-2**)

Compared to the gel-based methods, 1,742 unique proteins were identified from two independent biological samples subjected to MudPIT, using Mascot with false discovery rate (FDR) < 0.015 and Protein Pilot with FDR < 0.05 (see Material and methods). Protein identification acceptance criteria were C.I ≥ 98% for proteins identified with multiple peptides (1,491 proteins), and C.I ≥ 99.9% for proteins detected from a single peptide (251 proteins). In total, 1,764 unique Arabidopsis guard cell proteins were

identified from combined application of all methods (**Figure 2-1B** for summary, **Table 2-3** for the proteins identified from any two of the three methods and **Table 2-1** for all identified guard cell proteins).

**Global bioinformatic analyses of the guard cell proteome**

Enrichment of gene ontology (GO) (http://www.arabidopsis.org/tools/bulk/go/index.jsp) categories in the guard cell proteome as compared to the conceptually predicted whole Arabidopsis proteome was determined using the topGO package (Alexa et al., 2006; Baerenfaller et al., 2008). For the GO Cellular Component term, proteins localized in chloroplast thylakoid membranes and mitochondria are overrepresented in the guard cell proteome (**Figure 2-2A**). For the GO Biological Process term, the guard cell proteome is enriched in the GO categories "response to cold," "translation" and "glycolysis" (**Figure 2-3**, **Table 2-4**; for all enriched GO categories in the guard cell proteome, $P < 10E-6$). In particular, enzymes catalyzing all steps of glycolysis were present (**Figure 2-2B**).

The one kb regions upstream of the start codons of genes encoding the guard cell-identified proteins were analyzed by Motif analysis (http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp). Compared to the whole genome, GGCCCA and its complementary sequence TGGGCC are the most enriched regulatory motifs in guard cells (35.8% in the identified guard cell proteome vs 24.9% in the predicted whole Arabidopsis proteome). These motifs are also enriched in the promoters of nuclear genes encoding proteins of mitochondrial oxidative phosphorylation complexes (Welchen and Gonzalez, 2006).

Twelve proteins previously shown to play a role in guard cell function were present in our guard cell proteome (**Table 2-5A**). In addition, functional classification by GO analysis showed that 53 proteins out of our identified guard cell proteome are predicted as signal transduction proteins (**Table 2-5B**). Of these, only two proteins, PLD$\alpha$1 (Mishra et al., 2006) and CPK3/CDPK6 (Mori et al., 2006), have been previously

studied in the context of guard cell function. The remaining 51 proteins may prove fertile ground for deeper understanding of guard cell signaling.

To identify proteins in our guard cell proteome that may be specifically expressed or enriched in guard cells, we compared our guard cell proteome to ~13,000 proteins in previously identified proteomes including cell wall (Bayer et al., 2006), trichome (Wienkoop et al., 2004), epidermal cell (Wienkoop et al., 2004), and leaf (Lee et al., 2007a) proteomes, as well as to the recently reported proteome map of Arabidopsis organs (Baerenfaller et al., 2008). These comparisons revealed 71 proteins that were only identified in our guard cell proteome (**Table 2-6**). These 71 proteins may have unique roles in guard cell function, particularly since, to date, only 12 of these proteins have been shown to function in any other tissues by mutant analysis (**Table 2-6**). Surprisingly, of the proteins identified by Baerenfaller et al. (2008) as "biomarkers" of specific organ types, 11 of these proteins (three from flowers, three from siliques, four from roots, and one from seeds) were also discovered in our guard cell proteome (**Table 2-7**). While flowers and siliques have limited numbers of guard cells, roots and seeds do not have guard cells, thus this comparison strongly indicates the importance of evaluating single cell type proteomes in the context of biomarker identification.

## Functional analysis of the most abundant protein in guard cells, the myrosinase TGG1

The plant glucosinolate-myrosinase system is an effective defense system against bacteria, pathogens and herbivores. When tissue is damaged, e.g. by insect chewing, glucosinolates are thought to be released from the vacuole and hydrolysed by myrosinases into a variety of toxic small molecules, including thiocyanate, isothiocyanate and nitrile which are active against biotic intruders (Wittstock and Halkier, 2002; Barth and Jander, 2006). TGG1 is one of the two functional myrosinase enzymes expressed in Arabidopsis leaves, and reporter gene analysis has shown that *TGG1* but not *TGG2* is

highly expressed in guard cells; the only other locus of expression of these genes is in phloem idioblasts (Husebye et al., 2002; Barth and Jander, 2006).

The remarkable abundance of TGG1 in guard cells suggested that TGG1 has important roles in guard cells, but this hypothesis had not been previously investigated. Previous studies by Jander and colleagues showed that degradation of glucosinolates is slower in *tgg1* mutant leaves compared to wild-type Col leaves upon mechanical damage (Barth and Jander, 2006). We evaluated TGG1 functions in guard cells using *tgg1* mutant plants (Barth and Jander, 2006). *tgg1* mutants showed no obvious whole plant phenotypes or developmental defects, consistent with previous reports (Barth and Jander, 2006). We discovered that wounding induces stomatal closure and *tgg1* guard cells showed moderate disruption of this response (**Figures 2-4, Figure 2-5**).

Given that ABA regulation of stomatal movements is central to guard cell function and that TGG1 is the most abundant protein in guard cells, ABA regulation of stomatal apertures was evaluated in the *tgg1* mutants. *tgg1* mutant stomata showed wild-type responses to ABA-promotion of stomatal closure but were hyposensitive to ABA-inhibition of stomatal opening (**Figure 2-6A-B**). These results strongly suggest that TGG1 is a positive regulator in ABA-inhibition of stomatal opening and thus has a heretofore unrecognized role in plant abiotic stress responses.

In wild-type Col plants, ABA is known to inhibit the guard cell $K^+_{in}$ channels that mediate $K^+$ uptake during stomatal opening (Pandey et al., 2007) (**Figure 2-6C-D**). In the absence of ABA treatment, *tgg1* mutant guard cells had similar $K^+_{in}$ current amplitudes and kinetics as Col; however, ABA-inhibition of $K^+_{in}$ current was abolished in the two independent *tgg1* mutants, *tgg1-1* and *tgg1-3* (**Figure 2-6C-D**). Outward $K^+$ currents, which mediate $K^+$ efflux during stomatal closure, were statistically identical in Col vs. *tgg1* mutants either with or without ABA treatment (**Figure 2-7**).

Alterations in the glucosinolate profiles of *tgg1* mutants have already been characterized (Barth and Jander, 2006). Since myrosinases hydrolyze glucosinolates, one question which arises is, what is functioning in the ABA-inhibition of $K^+_{in}$ channels: myrosinase itself, glucosinolates, or the hydrolyzed products of glucosinolates? As a first step toward addressing this question, glucosinolates, or myrosinase, or a combination of

glucosinolates and myrosinase, was directly applied to the cytosol of Col and *tgg1* mutant guard cells via the patch pipette solution (**Figure 2-6**). Whole cell patch clamp data showed that glucosinolates administration resulted in inhibition of $K^+_{in}$ channels in Col guard cells but not in guard cells of *tgg1* mutants, indicating that the glucosinolates themselves do not suffice to inhibit channel activity. By contrast, co-administration of glucosinolates and myrosinase resulted in a similar extent of inhibition of $K^+_{in}$ channels in both Col and *tgg1* mutants, suggesting that it is the hydrolyzed products of glucosinolates that evoke channel inhibition. However, myrosinase addition alone had no effect on $K^+_{in}$ channels in either Col or *tgg1* mutants, suggesting that, in the absence of an appropriate triggering event, glucosinolate substrates are not available for myrosinase action. Most importantly, even though myrosinase application alone had no effect on $K^+_{in}$ currents, application of myrosinase restored $K^+_{in}$ sensitivity to ABA in *tgg1* guard cells (**Figure 2-6C-D**). These results indicate that the hydrolysis of glucosinolates by myrosinases is induced in some manner (see Discussion) by ABA in Arabidopsis guard cells, and is a necessary component of ABA-mediated $K^+_{in}$ channel inhibition.

**The role of glycolysis in stomatal movements**

Proteins involved in energy provision are enriched in guard cells (**Figure 2-2**). Of the 55 glycolytic proteins, 32 (58%) were identified in our guard cell proteome and they cover all the steps of glycolysis. Glycolysis is catalyzed by parallel pathways in the cytosol and plastids (Plaxton, 1996) and we identified glycolytic enzymes from both compartments: 11 of the 32 identified glycolytic proteins in our guard cell proteome are predicted to have chloroplast transit peptides by ChloroP (Emanuelsson et al., 1999) (**Figure 2-2B**). The interconversion of 3-phosphoglycerate and 2-phosphoglycerate is catalyzed in plants by a small gene family, the phosphoglycerate mutases (*PGMs)*. *PGM*s are divided into two classes depending on whether they need 2,3-biphosphoglycerate as a cofactor: independent *PGM*s which are mainly found in plants (Grana et al., 1995) and dependent *PGM*s (Jedrzejas, 2000). In the Arabidopsis genome there are two annotated

*iPGMs*, At1g09780 and At3g08590, encoding proteins with 90% sequence identity, and one "*iPGM* family" protein (previously annotated as a *dPGM*), At4g09520. This small gene family facilitates functional characterization by mutant analysis. The two iPGMs share 90% amino acid sequence identity. Both iPGMs were identified in our guard cell proteome: peptides unique to each isoform were identified from both BR and 2D LC-MALDI MudPIT methods. The 1000 base pair upstream regions of the start codons of the two *iPGM* genes contain the guard cell-enriched motif, TGGGCC. To date, the regulatory mechanisms of iPGMs remain unknown in any plant cell system.

To study the roles of iPGMs and their corresponding metabolic pathways in stomatal movements, we identified and characterized *ipgm1* and *ipgm2* T-DNA insertional mutants. Two independent T-DNA insertion alleles for each *iPGM* gene were identified (**Figure 2-8**). RT-PCR assay showed that both *ipgm1* alleles are null mutants, lacking full-length transcripts (**Figure 2-8B**). The two *ipgm2* alleles are knock-down mutants (**Figure 2-8C**): full-length transcripts of *iPGM2* are present at reduced levels in both *ipgm2* mutants as compared to Col (**Figure 2-8C**). Two fully independent double mutants (*ipgm1-1 ipgm2-2 = double 1*, *ipgm1-2 ipgm2-1 = double 2*) were obtained by crossing (**Figure 2-8E**). Enzymatic assays of iPGM activity (Westram et al., 2002; Bourgis et al., 2005) using five-week old leaves showed that all the single *ipgm* mutants have reduced iPGM enzyme activities as compared to wild-type (Col) but the rates are not significantly different except possibly for *ipgm2-2* ($P = 0.04$). Importantly, *ipgm* double mutants have no detectable iPGM enzyme activity (**Figure 2-8F**).

Since both iPGMs were found in the guard cell proteome, we initially focused on stomatal phenotypes. Blue light induces stomatal opening (Shimazaki et al., 2007) and previous analysis showed that blue light-stimulated stomatal opening was dimished in plants deficient in phosphoglucomutase, which catalyzes the conversion of glucose 1-phosphate to glucose 6-phosphate upstream of glycolysis (Lasceve et al., 1997). There is also evidence for light-activation of phosphofructokinase (PFK) or pyrophosphate: fructose 6-phosphate phosphotransferase (PFP) in fava bean guard cells (Hedrich et al., 1985). Accordingly, we evaluated blue light-promotion of stomatal opening in the *ipgm* mutants. Low $CO_2$ induction of stomatal opening under darkness was also studied since

stomatal opening under darkness is thought to mainly rely on energy from oxidative phosphorylation (Schwartz and Zeiger, 1984). ABA regulation of stomatal movement is also energy-requiring (Weyers et al., 1982) and was studied as well.

*ipgm* single mutants had similar phenotypes as Col in most stomatal aperture assays, suggesting functional redundancy of the two iPGM proteins. However, both independent double mutants were hyposensitive to blue light (*double1* P = 0.01, *double2* P = 0.008) and low $CO_2$ promotion of stomatal opening (*double1* P = 0.001, *double2* P = 0.009) (**Figure 2-9A-B**). ABA promotion of stomatal closure (*double1* P = 0.002, *double2* P = 0.006) and inhibition of stomatal opening (*double1* P = 0.002, *double2* P = 0.008) were both abolished in the double mutants (**Figure 2-9C-D**). *ipgm2-2* is hyposensitive to ABA promotion of stomatal closure (P = 0.003) consistent with its relatively lower iPGM enzyme activities (**Figure 2-8F**). Similar to the stomatal movement phenotypes, single *ipgm* mutants do not show any morphological phenotypes under either long or short day growth conditions (data not shown). However, the growth of the double mutants is dramatically reduced (**Figure 2-9E-F**). In addition, the *ipgm* double mutants are self-sterile and fail to produce visible pollen grains (**Figure 2-9G**).

## Discussion

Proteomics, an important post-genomic approach, has been applied to many fields, e.g. identification of protein expression profiles changes under stress conditions (Hashimoto and Komatsu, 2007), analysis of post-translational modifications (Kwon et al., 2006) and determination of protein-protein interactions (Parrish et al., 2007). Quantitative proteomic methods are also emerging, but such quantifications will have greatest correspondence to actual cellular protein amounts in the context of single cell type proteomes, as opposed to mixed tissues or organs where abundance of a given protein may vary greatly among the different cell types present. Although fava bean guard cells were used as material for an in-gel kinase assay ~a decade ago, leading to identification of a $Ca^{2+}$-independent ABA-activated protein kinase (AAPK) by mass

spectrometry (Li et al., 2000), a major bottleneck for the characterization of the guard cell proteome has been obtaining enough highly pure guard cell from a species with a sequenced genome. Here, this bottleneck was overcome and three proteomics methods were used to identify 1,764 unique proteins of the guard cell proteome.

**Comparison of the three proteomic methods**

In our study, the two gel-based methods (BR and NR) yielded similar numbers of identified proteins, while the gel-free method (2D LC-MALDI MudPIT) had an identification rate ~30 fold higher than those from the gel-based methods. Twenty-one proteins, i.e. approximately a third of the proteins identified by BR or NR methods, were common to both of these gel-based approaches. Eight-eight percent of the BR proteins (51 proteins) and 71% of the NR proteins (42 proteins) were also identified by 2D LC-MALDI MudPIT analyses. Only 19 proteins (representing 33% of the BR proteins, 32% of the NR proteins, and 1% of the 2D LC-MALDI MudPIT proteins) were found from all three methods (**Figure 2-1B**, **Table 2-3**). This low overlap is consistent with observations from other organisms which have shown that multiple strategies are required to obtain high coverage of the proteome (Kleffmann et al., 2007). The limitations of gel-based methods regarding identification of basic, high molecular mass, and membrane proteins are well known (Rossignol et al., 2006). However, 22 proteins of the guard cell proteome were exclusively identified by gel-based methods, and gel-based methods provide more reliable inference of post-translational modifications (**Table 2-2**) indicating that gel-free methods cannot completely replace traditional gel-based methods (Lambert et al., 2005).

**Comparison of the guard cell proteome with other proteomes**

Although we used GCPs as starting material, five of the identified guard cell proteins are predicted to be cell wall proteins by GO software: calnexin 1 (CNX1,

At5g61790), defective glycosylation 1 (DGL1, At5g66680), At2g01720, At3g57030 and leucine rich repeat protein (LRR1, At5g16590). Two of these five proteins, At3g57030 and LRR1, were identified by a previous cell wall proteomic study (Bayer et al., 2006): these proteins may localize to multiple subcellular compartments including both apoplastic and a symplastic destinations, or be present in secretory vesicles that have not yet fused with the plasma membrane (Lee et al., 2004). Indeed, further GO analysis predicted that four of the five proteins (CNX1, DGL1, At2g01720, At3g57030) localize to the endoplasmic reticulum (ER), with the fifth protein, LRR1, localizing to the plasma membrane.

Of the 71 proteins that were identified in our guard cell proteome (**Table 2-6**) but not in other known proteomes, some may be specific to guard cells and thus can be considered as guard cell biomarkers. In this regard, it would be of particular interest to characterize the ten proteins of unknown function (**Table 2-6**). Others may be more abundant in guard cells than elsewhere and thus we succeeded in identifying these proteins as part of the guard cell proteome while they were missed in other proteomic analyses, e.g. the likely G-protein coupled receptor, GCR1, which confers ABA hypersensitivity to both stomatal movements and root growth (Pandey and Assmann, 2004). Conversely, our identification in the guard cell proteome of proteins previously thought to be biomarkers for roots and seeds (**Table 2-7**) indicates the importance of single cell type proteome analysis in determinations of protein distribution.

Five out of these 71 proteins are predicted to be signal transduction proteins by GO analysis: four protein kinases (At1g73670, At3g18040, At4g28650, At5g53320) and CLE5 (At2g31083). Some CLE proteins have been shown to be intercellular signals and ligands for surface receptor proteins involved in the control of cellular differentiation (Mitchum et al., 2008), but functions of any CLE proteins in guard cell development or stomatal physiology have not been shown to date.

**The guard cell proteome and guard cell signaling**

Twelve out of the 67 proteins previously identified as functioning in Arabidopsis guard cells/GCPs (**Table 2-8**) were present in our guard cell proteome (**Table 2-5A**); the other 55 may be low abundance proteins, or induced under specific conditions. The identified proteins are mainly involved in light (PHOT1, PHOT2 and AtCHX20) and ABA (GCR1, OST2, NIA2 and PLDα1) signaling (Fan et al., 2004; Israelsson et al., 2006; Shimazaki et al., 2007).

Of the 53 proteins categorized as signal transduction proteins by GO analysis (**Table 2-5B**), 51 have yet to be studied in the context of guard cell function, highlighting the usefulness of proteome analysis in identifying targets for further functional analyses. Thirteen are protein kinases, including one CDPK (CPK3/CDPK6), seven LRR protein kinases and five MAP kinases. This strongly suggests that phosphorylation is one of the main post-translational modifications in guard cells, consistent with previous demonstrations of the importance of phosphorylation to light and ABA signaling in guard cells (Li et al., 2000; Israelsson et al., 2006; Mori et al., 2006; Shimazaki et al., 2007). Five of the 53 are involved in auxin signaling, suggesting that auxin may be more important in guard cell physiology than previously recognized.

Based on microarray analyses with an Affymetrix chip representing ~8,000 transcripts Leonhardt et al. (2004), identified 1,309 transcripts as expressed in guard cells; of these, 64 were preferentially expressed in guard cells by comparison with their leaf transcriptome. Of the 64 transcripts, only 15 of their encoded proteins were identified in our guard cell proteome, and none of the 64 proteins are found solely in the guard cell proteome (**Table 2-9**). The remaining 49 may be low abundance proteins or show significant post-transcriptional regulation. On the other hand, 1,438 proteins were present in our guard cell proteome that were not present in Leonhardt et al.'s transcriptome (Leonhardt et al., 2004), while 294 proteins were present in our guard cell proteome that were not represented in an (unreplicated) guard cell transcriptome study using the "full genome" ATH1 chip (Yang et al., 2008). In addition, 46 proteins were present in our proteome that are not represented on the ATH1 chip, which represents ~22,000 of the

current 32,900 gene models. Clearly, proteomic studies can thus provide information not obtainable from microarray analyses.

**TGG1 function in guard cells**

TGG1 comprises 40-50% of the total protein in guard cells, a percentage comparable to that found for the primary carboxylation enzyme, rubisco, in mesophyll cells (Evans, 1989). However, no roles for TGG1 in guard cells had been previously demonstrated. We showed that *tgg1* mutant plants lacking this enzyme were unresponsive to ABA-inhibition of stomatal opening and $K^+_{in}$ channel regulation. Intracellular application of myrosinase alone did not restore $K^+_{in}$ channel inhibition in *tgg1* guard cells or cause channel inhibition in wild-type guard cells, yet application of myrosinase in the presence of ABA restored channel inhibition to the *tgg1* mutants, consistent with the following scenario: 1) myrosinase and its substrates, the glucosinolates, are localized in distinct subcellular compartments in guard cells, as has been proposed for other cell types (Grubb and Abel, 2006). 2) ABA induces the re-localization of glucosinolates to the cytosol. 3) The hydrolysis of available glucosinolates by TGG1 leads to inhibition $K^+_{in}$ channels in guard cells, and this is one component of ABA-inhibition of stomatal opening. Alternatively, ABA signaling might somehow increase the activity of myrosinase, or its affinity for its substrate. ABA regulation of glucosinolate compartmentalization, as proposed here, could help to explain non-defensive developmental changes in glucosinolate concentrations (Petersen et al., 2002; Brown et al., 2003) which occur in the absence of the tissue disruption that brings substrate and enzyme together during herbivory. Conversely, given the mechanisms described here, wound-induced increases in ABA reported to occur in leaf tissue (Schmelz et al., 2003) might provide positive feedback to the glucosinolate-myrosinase defense pathway.

Since application of glucosinolates to wild-type guard cells, or application of glucosinolates plus myrosinase to *tgg1* mutant guard cells, also inhibits the $K^+_{in}$ channels, we infer that it is the reaction catalyzed by myrosinase, e.g. the hydrolyzed products of

glucosinolates, that evoke channel inhibition. Alteration in membrane potential is a rapid response to wounding (Maffei et al., 2007), and our observations now suggest that $K^+$ channel regulation may contribute to these early electrical events. As the inward $K^+$ channels of guard cells are, on a sequence homology basis, most similar to metazoan Shaker channels (Pandey et al., 2007), animal Shaker channels may also be targets for these plant secondary compounds, which are known to have both toxic and anti-carcinogenic effects in mammals (Halkier and Gershenzon, 2006).

Further research is required to determine the mechanistic basis of $K^+_{in}$ channel regulation. It is noteworthy that recent studies have shown that isothiocyanates alter activity of the mammalian pain-sensing TRP1A channels by covalent modification at cysteine residues (Hinman et al., 2006). Alternatively, or in addition, the fact that myrosinase binds the antioxidant, ascorbic acid, and can catalyze formation of a condensation product of ascorbic acid with methylindoles (Burmeister et al., 2000) may suggest that myrosinase activity promotes ABA signaling via decreasing the ability of the guard cell to scavenge ROS. ROS elevation is a key signal transduction element in ABA signaling and plants engineered for increased ascorbate levels exhibit decreased levels of ROS and decreased guard cell responsiveness to ABA (Chen and Gallie, 2004).

While it was previously demonstrated that small molecules such as NO and ROS are shared between ABA and defense signaling pathways, including in guard cells (Melotto et al., 2006; Ali et al., 2007), enzymes of secondary metabolism such as TGG1 have not previously been implicated in ABA signaling. The interconnection discovered here between ABA and the glucosinolate-based biotic defense mechanism suggests a mechanism whereby exposure to abiotic stresses may enhance plant defense against subsequent biotic invaders. One general property of the hydrolyzed products of glucosinolates is volatility (Yan and Chen, 2007). The extremely high abundance of TGG1 in guard cell might facilitate evaporation of the hydrolyzed products from stomatal pores and thus maximize both deterrence of would-be herbivores and attraction of their parasites and predators (Bradburne and Mithen, 2000), as well as possibly initiate between- or within-plant defense signaling mechanisms (Baldwin et al., 2006; Frost et al., 2007).

Low doses of the products of glucosinolate hydrolysis can block tumor initiation in human tissues, and vegetables containing glucosinolates are reported to be helpful in cancer prevention (Halkier and Gershenzon, 2006). Pre-harvest growth conditions, harvesting processes, and storage conditions all can affect glucosinolate concentrations (Johnson, 2002). The implication from our data that ABA may regulate the sequestration and stability of plant glucosinolates may provide valuable information regarding optimal agronomic protocols to enhance human health.

**Glycolytic enzymes are important to guard cell function**

Both stomatal opening and stomatal closure, including ABA-induced stomatal closure, require energy (Weyers et al., 1982; Schwartz and Zeiger, 1984; Assmann and Zeiger, 1987). The enrichment of proteins localized in the chloroplast thylakoid membrane and in mitochondria (**Figure 2-2A**) in our guard cell proteome, is indicative of important roles for both photophosphorylation and oxidative phosphorylation in guard cells (Schwartz and Zeiger, 1984; Vavasseur and Raghavendra, 2005). Previous pharmacological studies using the photosynthetic inhibitor, 3(3,4-dichlorophenyl)-l,l-dimethylurea (DCMU), and the respiratory poison, KCN, suggested that stomatal opening under light mainly relies on photophosphorylation as a source of ATP, while stomatal opening induced by low $CO_2$ under darkness mainly relies on oxidative phosphorylation (Schwartz and Zeiger, 1984).

Our *in silico* analyses suggested an important role for glycolysis in stomatal function. We assessed and validated this prediction by wet-bench analyses. The hyposensitive phenotypes of *ipgm* mutants in low $CO_2$-induction of stomatal opening under darkness and blue light promotion of stomatal opening (**Figure 2-9A-B**) provide the first genetic evidence that glycolytic enzymes are critical for guard cell function in response to environmental signals. ATP can be generated via glycolysis under darkness (Plaxton, 1996) and malate$^{2-}$, an important guard cell osmoticum and counter-ion for $K^+$, can be produced through glycolysis under blue light in plants (Shimazaki et al., 2007).

Therefore, our results suggest that glycolysis regulates stomatal movements via energy provision under darkness and malate$^{2-}$ synthesis under blue light. The ABA hyposensitivity of double *ipgm* mutants suggests that glycolysis contributes to the energy requirements for ABA-regulated stomatal movements (Weyers et al., 1982). 3-PGA is the primary carboxylation product fava bean guard cells (Gotow et al., 1988), and 3-PGA production is inhibited by ABA (Popova et al., 1987). 3-PGA accumulated in potato plants when iPGM enzyme activity was reduced by antisense inhibition (Westram et al., 2002). Therefore, an alternative hypothesis for the ABA hyposensitivity phenotype of double *ipgm* mutants is that resultant high concentrations of 3-PGA in guard cells prevent decreases in 3-PGA levels that would normally be induced by ABA. However, if and how 3-PGA regulates stomatal movements awaits further investigation.

Given the severe growth phenotype of the *ipgm* double mutants (**Figure 2-9E-F**), a function of dPGM(s) in glycolysis in Arabidopsis remains questionable. The failure of double *ipgm* mutants to produce pollen may indicate high energetic requirements for pollen production, consistent with the fact that many male sterility mutants are associated with disruptions of genes encoding mitochondrial proteins (Chase, 2007).

**Concluding remarks**

Since guard cells are present in all vascular plants and are critical in protecting plants from abiotic and biotic stresses, identification of the guard cell proteome is a crucial contribution toward elucidation of how plants interact with the local climate and biotic environment. The guard cell is a pre-eminent model system in plant cell biology, and the discovered guard cell proteome can be used to inform the reconstruction of guard cell signaling networks *in silico* (Li et al., 2006) and *in planta*. In particular, our demonstration of TGG1 involvement in ABA signaling highlights an interplay between biotic and abiotic stress responses in plants. The strategy applied here, beginning with global protein identification within a single cell type and ending with discovery of novel

signaling pathways by functional analysis of the identified proteins, demonstrates a powerful approach for future single cell type studies in plants and metazoans.

## Material and methods

### Plant material, GCP isolation and purity

Seeds were surface sterilized with 70% ethanol for five min, washed with 95% ethanol three times for one min., allowed to dry, and then plated on ½ MS plates containing 1% agar, 1% sucrose, pH 5.7. All plates were placed at 4$^{o}$C for two days for stratification under darkness and then kept for eight days in a growth chamber with eight hour short day conditions, 110 μmol $m^{-2}s^{-1}$ of light, 75% humidity and 19$^{o}$C temperature. All healthy seedlings were transplanted into soil (potting mix, Miracle-Gro, Inc.) and grown in the same growth chamber for 3-4 more weeks.

Healthy rosette leaves were harvested from five week old plants. GCPs were isolated using the same day two-step enzyme digestion method (Pandey et al., 2002). The main contamination for GCP isolations comes from mesophyll cell protoplasts, so we discarded any GCP isolations with >1% mesophyll cell protoplast contamination. After obtaining the guard cell proteome, we further evaluated the issue of contamination by comparing the Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) protein amount in leaves and GCP. Rubisco is a key enzyme in the Calvin cycle and accounts for >50% of the total leaf protein (Evans, 1989). However, in our proteomic studies, the most abundant protein spot on 2D gels is TGG1 in both BR and NR methods and Rubisco protein spots are hardly seen (**Figure 2-1C-D**, **Table 2-1**). This result not only indicates that the protein contamination from chloroplasts and chloroplast-rich mesophyll cells is low, but also is consistent with previous indications that photosynthethic carbon fixation is not a major function of guard cell chloroplasts (Shimazaki, 1989).

**Protein extraction and separation for MS analysis**

For the BR analysis, 10 µl of urea/thiourea lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris, 4% w/v CHAPS) was added per million GCPs, then GCPs were vortexed for 30 min in a 4$^{o}$C cold room. After centrifugation at 12,000 g for 10 min, the supernatant was removed and proteins were precipitated overnight at -20$^{o}$C by adding three volumes of ice-cold TCA (10% TCA, 0.05% DTT in acetone). On the second day, the sample was centrifuged at 12,000g for 10 min, and the precipitate was washed three times with ice-cold acetone including 0.05% DTT. Finally, the pellet was vacuum-dried for 30 min and dissolved in 135 µl urea/thiourea lysis buffer (same as above). Protein concentration was determined by Bio-Rad protein assay (Cat #: 500-0006). One mg total protein was loaded in a pH 3-10, 24 cm IPG strip (Bio-Rad) which was rehydrated overnight with 450 µl rehydration solution (8 M urea, 2% CHAPS, 2% IPG buffer, 20 mM DTT, 0.001% bromophenol blue). Samples were cup-loaded in IPG strips which were run in a Multiphor II system (Pharmacia Biotech) with the conditions 500 V (gradient) for 1 min, 3500 V (gradient) for 1.5 hours and 3500 V for 10.5 hours. The second dimension was run in a Bio-Rad Protean II Xi cell with the condition 45 mA for 4.5 hours. All 2D gels from this method were stained with Colloidal Coomassie Blue. Two independent biological replicates were performed.

For prefractionation with IEF fractionator (Invitrogen, Cat #: ZF10001), total protein was extracted from 50 million GCPs per sample according to the IEF fractionator manual. GCPs were ground into fine powder under liquid nitrogen. The powder was treated overnight at -20$^{o}$C by adding 1 ml of ice-cold TCA. On the second day, the sample was centrifuged at 16,000g for 20 min, and the pellet was washed with ice-cold acetone until colorless. Finally, the sample was vacuum-dried for one hour and dissolved in 950 µl IEF denaturant (7.7 M urea, 2.2 M thiourea, 4.4% CHAPS, deionized with exchanger resin (Sigma Cat #: A-5710)) plus 10 µl 2 M DTT and protease inhibitor. Protein concentration was determined by Bio-Rad protein assay. One mg total protein from each replicate was prefractioned into five different pH ranges by six pH discs (3, 4.6, 5.4, 6.2, 7.0, 10.0) using the IEF fractionator. The running conditions were 100 V for

20 min; 200 V for 80 min and 600 V for 120 min; the final current was 0.3 mA. Each fractionation was precipitated with three volumes ice-cold 13.3% TCA, 0.093% β-mecaptoethanol in acetone overnight at -20$^{o}$C. On the second day, the sample was centrifuged at 16,000g for 20 min, and the pellet was washed three times with ice-cold acetone containing 0.07% β-mecaptoethanol. After washing, the pellet was vacuum-dried for 30 min and dissolved in 160 µl rehydration buffer (8 M urea, 2% CHAPS, 0.001% bromophenol blue, 10 µl 2 M DTT and 20 µl Zoom carrier ampholytes pH 3-10). The same buffer was also used to rehydrate seven cm IPG strips overnight at room temperature. The IPG strips had similar pH ranges as the pH fractionations from the IEF fractionator: pH 3-6 IPG strips (Bio-Rad) were used for pH 3-4.6 fractions, pH 4.5-5.5, 5.3-6.3, 6.1-7.1, 6-10 strips (Invitrogen) were used for pH 4.6-5.4, 5.4-6.2, 6.2-7.0, 7.0-10.0 fractions respectively. IPG strips were run with the conditions 175 V (gradient) for 15 min, 2000 V (gradient) for one hour and 2000 V for six hours. The second dimension was run in a Bio-Rad mini-protean cell system with the conditions 100 V for 10 min, then 200 V for 45 min. All the 2D gels from this method were stained with Sypro-Ruby. Two independent biological replicates were analyzed.

For LC-MALDI MudPIT analyses, for each independent biological sample, total protein was extracted from 50 million GCPs using the same method as described for IEF fractionation. Proteins were in-solution digested according to the method of Adachi et al. (2006). One mg total protein was denatured and reduced in 50% trifluoroethanol (TFE) and 50% 20 mM $NH_4HCO_3$ (pH~8.0) with 10 mM DTT at 90$^{o}$C for 30 min. Then the sample was alkylated by adding IAA (iodoacetamide) to 50 mM final concentration and incubated for one hour at RT in the dark. Alkylation was stopped by quenching the excess IAA by adding DTT to 10 mM final concentration. The solution was further incubated at RT for 1 hour in the dark. The final concentration of TFE was diluted to 5% with 20 mM $NH_4HCO_3$ (pH 8.0). ~30 µg trypsin was added to the sample (protein:trypsin=33:1) for digestion at 37$^{o}$C for 18 hours. Digestion was stopped by adjusting pH to 3~4 with trifluoroacetic acid (Adachi et al., 2006). Two independent biological replicates were done.

Each proteomic method (BR, NR, 2D LC-MALDI MudPIT) was applied to two independent biological samples, i.e. a total of six samples and a total of ~3 x$10^8$ GCPs

**Spot cutting, trypsin digestion and spotting on MALDI plates**

All consistently visible spots in both BR 2D gels were cut manually and all consistently detected spots in both NR 2D gels were cut using a spot-cutter (Bio-Rad EXQuest spot cutter). All spots were digested with trypsin (Promega) according to a standard protocol from the Hershey Medical Center Mass Spectrometry Facility (http://www.hmc.psu.edu/core/oldsite/Maldi/malditofprotocols.htm), desalted with SCX Ziptips following the ZipTip (Millipore, Cat #: ZTSCXS096) instruction manual, and then spotted on MALDI plates. After the samples were dried, each spot was overlaid by 0.6 µl of matrix solution (5 mg/ml of *a*-cyano-4-hydroxycinnamic acid (CHCA), 2 mg/ml of ammonium phosphate, 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile).

**MS and data analysis**

All protein spots were analyzed using a 4700 or 4800 proteomic analyzer MALDI-TOF/TOF tandem system (Applied Biosysems). For 2D LC MALDI MudPIT analysis, all the trypsin-digested peptides were separated using two sequential separation methods, strong cation exchange (SCX) and C18 nanoflow chromatography. Each sample was first separated into 15 fractions using SCX chromatography performed on a passivated Waters 600E HPLC system, using a 4.6 X 250 mm PolySULFOETHYL Aspartamide column (PolyLC, Columbia, MD) at a flow rate of 1 ml/min. Buffer A contained 10 mM ammonium formate, pH 3.6, in 20% acetonitrile/80% water. Buffer B contained 666 mM ammonium formate, pH 3.6, in 20% acetonitrile/80% water. The gradient was Buffer A at 100% (0-30 minutes following sample injection), 0%→ 35% Buffer B (30-48 min), 35→100% Buffer B (48-49 min) 100% Buffer B (49-56 min), then

at 56 min switched back to 100% A to re-equilibrate for the next injection. The first 28 ml of eluant (containing all flow-through fractions) was combined into one fraction, then 14 additional 2-ml fractions were collected. All 15 of these SCX fractions were dried down completely to reduce volume and to remove the volatile ammonium formate salts, then resuspended in 15 µl of 2% (v/v) acetonitrile, 0.1% (v/v) TFA. Any particulates were filtered out by centrifuging through a 30 µl aerosol barrier tip (Denville Scientific) contained in a 1.5 ml microfuge tube, prior to reverse phase C18 nanoflow-LC separation. Each SCX fraction was autoinjected onto a Chromolith CapRod column (150 X 0.1 mm, Merck) using a five µl injector loop on a Tempo LC MALDI Spotting system (ABI-MDS/Sciex). Buffer C was 2% acetonitrile, 0.1% TFA, and Buffer D was 98% acetonitrile, 0.1% TFA. The elution gradient was 100% C (0-4 min), 0→10% D (4-10 min), 10%→25% D (10-30 min), 25%→40% D (30-35 min), 40%→80% D (35-38 min), 80% D (38-42 min), 80%→0% D (42-43 min), 0% D (43-50 min). Flow rate was 3 µl/min, and an equal flow of MALDI matrix solution was added post-column (7 mg/ml recrystallized CHCA, 2 mg/ml ammonium phosphate, 0.1% TFA, 80% acetonitrile).

The combined eluant (column eluant plus matrix) was automatically spotted onto a stainless steel MALDI target plate once every six seconds (0.6 µl per spot), for a total of 370 spots per original SCX fraction, for a total from the 15 MALDI plates of 5,500 MALDI spots. This combined LC-MALDI procedure allowed us to run the MS/MS analyses after the entire plate has had MS spectra collected, thus allowing selection of the largest peak of each MS mass species observed for subsequent fragmentation and MS/MS analysis.

Two different software packages were used to analyze MS and MS/MS data, GPS Explorer (Applied Biosystems/MDS Sciex), using as an underlying search algorithm a locally-installed copy of the Mascot software programs, version 2.1 (Matrix Science http://www.matrixscience.com), and Protein Pilot software Version 2.0 (Applied Biosystems/MDS Sciex, using the Paragon algorithm (Shilov et al., 2007) for searching and the ProFound algorithm for protein inference and grouping from MS/MS spectral/peptide data). All MS and MS/MS data obtained from gel-based methods were analyzed using GPS Explorer, with a data-dependent analysis of the ten largest MS peaks

from each gel spot being automatically chosen for subsequent MS/MS analysis. Candidate protein IDs from individual gel spots were accepted if they had a GPS Explorer protein C.I > 99.5% (equivalent to a Mascot Score of $p < 0.005$) for both the peptide mass fingerprint (PMF) data arising from the MS spectrum and at least one MS/MS fragment ion spectrum.

MS/MS data from 2D LC MALDI MudPIT experiments were analyzed using both Mascot and Protein Pilot software version 2.0. For both algorithms, protein identification acceptance criteria were C.I ≥ 98% (equal to a Protein Pilot Unused Score of 1.7) for proteins identified with multiple peptides, and C.I ≥ 99.9% for proteins detected from a single peptide, plus acceptable FDRs. Although there is not yet a consensus on a standard way to do this, there is clear agreement in the field of proteomics that in addition to the use of expectation or p-value scores for individual proteins identified in large MudPIT-type experiments, paring down a list of identified proteins to the minimal list that accounts for all the peptide spectral data (avoiding redundant IDs and use of the same MS/MS spectral evidence to support the "identification" of multiple homologous proteins, sometimes called the "Protein Inference Problem") and a calculation of the FDR for the overall ID list are both crucial (Tabb, 2008).

In the Protein Pilot analyses, identified proteins from 2D LC MALDI MudPIT were accepted only if they met our C.I. criterion and also had an estimated FDR < 0.05. The decisions about how to arrive at the minimal protein list which accounts for all the observed spectral evidence are calculated by the ProFound algorithm also contained in the Protein Pilot software. The Unused Score for any identified protein in the Protein Pilot search results represents the summary of all the individual peptide scores which could be assigned to that protein only; this evidence is not shared with any higher ranking protein homologs or other higher ranking proteins. Thus, in the Unused Scores each MS/MS spectrum can only be used as evidence for one specific peptide and protein (the Total Score, which is not used in our analyses, in contrast, represents the summary of all spectral information that could be assignable to any particular protein). All identified proteins had Unused Score of 1.7 or higher, which corresponds to a confidence of 98% or higher. A second requirement was that all identified proteins have an estimated local

FDR of 5% or less, based on the number of IDs at any cutoff Unused Score from a "normal" database (NCBInr version of 4/17/07) compared to the number of IDs from a concatenated forward and decoy database plus a list of common potential contaminants such as keratins, common laboratory reagents such as BSA, and trypsin autolysis peaks. The decoy database is a randomized version of the same NCBInr database, where amino acid frequencies in the database are the same as in the normal database. The use of identifications of proteins in decoy databases to estimate FDR is described in Elias and Gygi, (2007). The FDR used as a cutoff for accepting Protein Pilot identified proteins was a local (sometimes called "instantaneous") FDR of 5% or lower, meaning that the protein with the lowest accepted score still had an estimated probability of less than 5% of being a false positive based on the rate of increase in the accumulation of decoy database hits at that particular cutoff score. This local FDR was calculated using a pre-release version of the Proteomics System Performance Evaluation Pipeline (PSPEP) tool (Tang et al., 2008).

In GPS Explorer/Mascot IDs, for ease of calculation, the more commonly used Total FDR (sometimes called "aggregate FDR"). Total FDR estimates are calculated by taking twice the number of decoy database hits at any cutoff score, and dividing by the total number of IDs. Thus, the FDR estimate applies to the entire set of identified proteins, e.g., a Total FDR estimate of 5% would mean that 5% of the identified proteins on the list are likely to be false positives. It is intuitively obvious that the lower scoring proteins at the bottom of the list are much more likely to be false positives than are the high-scoring proteins at the top of the list; thus, if the overall accepted FDR were to be set at 5%, the proteins at the bottom of the list would themselves have a higher than 5% probability of being false positives. For this reason, we set the acceptable Total FDR for the GPS Explorer/Mascot analyses at a more stringent 1.5%.

Search criteria for GPS Explorer/Mascot analyses were set up as follows: trypsin cleaved peptides; 1 missed cut allowed; 100 ppm peptide tolerance; carboxyamidomethylation as a fixed modification, and oxidized methionine, deamidation of Asn and Gln residues, and pyro-Glu formation from N-terminal Gln as variable modifications. Search criteria for Protein Pilot searches were trypsin-cleaved peptides;

iodoacetamide-modified cysteines; ID Focus = Biological Modifications; and Detected Protein Threshold = 0.05 (10.0%). Protein Pilot automatically searches for a series of potential biological and sample preparation-induced modifications once a suitable sequence tag of 3-4 amino acids has been found within an MS/MS spectrum. All the GI#s from the search results were converted to At locus identifiers, and the redundant proteins were eliminated.

All MS and MS/MS data obtained from gel-based methods were analyzed using GPS Explorer. Candidate protein IDs from individual gel spots were accepted if they had a GPS Explorer protein C.I > 99.5% (equivalent to a Mascot Score of $P < 0.005$. MS/MS data from 2D LC MALDI MudPIT experiments were analyzed using both Mascot and Protein Pilot software version 2.0. For both algorithms, protein identification acceptance criteria were C.I ≥ 98% (equal to a Protein Pilot Unused Score of 1.7) for proteins identified with multiple peptides, and C.I ≥ 99.9% for proteins detected from a single peptide, plus acceptable FDRs.

**Mutant identification**

*tgg1* mutants were generously provided by Dr. Georg Jander, Cornell University. Seeds of T-DNA insertion lines (SALK_003321 (*ipgm1-1),* SALK_029822 (*ipgm1-2*), SALK_016231 (*ipgm2-1*), SALK_002280 (*ipgm2-2*)) were acquired from the Arabidopsis Biological Resource Center (ABRC). Gene specific primers, T-DNA left border specific primers and T-DNA right border specific primers were used to genotype these lines (**Table 2-10** for primer information). Sequences flanking both sides of the T-DNA insertion sites were amplified from genomic DNA by PCR and PCR products were sequenced to confirm the precise T-DNA insertion sites. RNA was extracted using Trizol (Invitrogen). RT-PCR was performed using SuperScript III (Invitrogen). *tgg1* mutants were generously provided by Dr. Georg Jander, Cornell University.

**iPGM enzymatic assay**

Healthy leaves from Col, single and double *ipgm* mutant plants were cut and frozen in liquid nitrogen. Total proteins were extracted and the iPGM enzyme assay was performed according to Bourgis et al. (2005). Chemicals were from Sigma.

**Stomatal aperture measurement**

Leaves were harvested from five week old healthy Arabidopsis plants just before initiation of the photoperiod for stomatal opening assays, and after the lights had been on for five minutes for stomatal closure measurements. Excised leaves with abaxial side down were placed in a 6-well petri dish containing five ml solution in each well. The solution for assays of stomatal opening was 10 mM KCl, 7.5 mM IDA, 10 mM MES pH 6.15 with KOH. The solution for assays of stomatal closure was 20 mM KCl, 5 mM MES, 1 mM $CaCl_2$, pH 6.15 with KOH.

For stomatal opening, the dish was placed in darkness for 2 hrs to promote stomatal closure and for the closure assays, the leaves were placed under light (200 µmol $m^{-2}s^{-1}$) for 2 hrs to induce stomatal opening. Five µl of 50 mM ABA (50 µM final concentration) or 100% ethanol (solvent control) was then added in each well for ABA treatment or control, respectively and leaves were further left under the light for 2 more hours for both ABA and control treatments.

For blue light treatment, leaves were put under darkness for two hours. Stomatal apertures were measured at this time to obtain a baseline, and the remaining leaves were transferred to blue light (10 µmol $m^{-2}s^{-1}$) for an additional three hours. For low $CO_2$ treatments, 200 ml of opening solution in a 600 ml beaker was pre-bubbled over night with $CO_2$ free air using the soda lime of a LICOR 6400 photosynthesis system to scrub $CO_2$ from the air. The flow rate of the $CO_2$ free air was set at 500 µmol/s overnight and then reduced to 200 µmol/s upon addition of the leaves. Before low $CO_2$ treatment, all leaves were put in the opening solution under darkness for two hours. Then control leaves

were left in the untreated opening solution and treatment leaves were transferred to the low $CO_2$ opening solution under darkness. Stomatal apertures were measured after an additional 2.5 hours.

For all aperture experiments, two leaves were used for each treatment except for blue light and low $CO_2$ experiments, where one leaf was used so that measurement time was minimized. To avoid the possible low confidence caused by one leaf per replicate, more replicates were performed for blue light and low $CO_2$ experiments. Five and eight independent replicates were performed for the blue light and low $CO_2$ experiments respectively, while three and four replicates were performed for ABA-regulation of stomatal aperture experiments in *tgg1* and *ipgm* mutants, respectively. Epidermal peels were prepared and ten epidermal images were photographed per leaf. At least 50 stomatal apertures were measured per leaf. All stomatal apertures were measured using Image J software.

**Electrophysiology (performed and text provided by Dr. Wei Zhang)**

Arabidopsis guard cell protoplasts were isolated as previously described (Wang et al., 2001). Standard whole-cell $K^+$ current recording was performed using solutions and protocols as defined by Coursol et al. (Coursol et al., 2003). Recordings obtained 10 min after attainment of the whole-cell configuration were used for the analysis; no significant rundown was observed for up to 40 min of recording in controls. Time-activated currents were defined as the average steady state current between 3.60 to 3.88 s minus the instantaneous current at 20 ms, and were normalized by whole cell capacitance to compensate for any variations in cell size.

For ABA treatment, 50 μM ABA was added in basic solution for $\geq$ 1.5 hours pretreatment of GCP and the same concentration of ABA was also present in the bath solution during patch clamping. For glucosinolate and myrosinase treatments, final concentrations of 50 μM total glucosinolates (Sigma ERMBC367) and/or 0.2 U/ml myrosinase (Sigma T45280) were added (from 50 mM and 50 U/ml stock solution for

glucosinolates and myrosinase respectively) into the pipette solution immediately before the start of the experiment. Glucosinolates were extracted according to the protocol provided (Sigma). $K^+$ current magnitudes were compared with Student's t-test; results with $P \leq 0.01$ were considered significantly different.

**ACKNOWLEDGEMENTS**

The College of Medicine Mass Spectromety and Proteomics Facility and the Proteomics and Mass Spectrometry Core Facility at University Park, Penn State University are gratefully acknowledged. We thank Dr. Georg Jander for *tgg* mutant seeds, and Profs. Hong Ma, Daniel Jones, and Jiaxu Li for comments on the manuscript. This work was supported by NSF grants MCB-0209694 and 6-2066-01 and USDA grant 2006-35100-17254 to SMA.

**References**

**Adachi, J., Kumar, C., Zhang, Y., Olsen, J.V., and Mann, M.** (2006). The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol **7,** R80.

**Alexa, A., Rahnenfuhrer, J., and Lengauer, T.** (2006). Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. Bioinformatics **22,** 1600-1607.

**Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsaltas, D., Leng, Q., von Bodman, S., and Berkowitz, G.A.** (2007). Death don't have no mercy and neither does calcium: Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell **19,** 1081-1095.

**Allen, G.J., Kuchitsu, K., Chu, S.P., Murata, Y., and Schroeder, J.I.** (1999). Arabidopsis abi1-1 and abi2-1 phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. Plant Cell **11,** 1785-1798.

**Allen, G.J., Chu, S.P., Harrington, C.L., Schumacher, K., Hoffmann, T., Tang, Y.Y., Grill, E., and Schroeder, J.I.** (2001). A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature **411,** 1053-1057.

**Allen, G.J., Chu, S.P., Schumacher, K., Shimazaki, C.T., Vafeados, D., Kemper, A., Hawke, S.D., Tallman, G., Tsien, R.Y., Harper, J.F., Chory, J., and Schroeder, J.I.** (2000). Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in Arabidopsis *det3* mutant. Science **289,** 2338-2342.

**Amme, S., Rutten, T., Melzer, M., Sonsmann, G., Vissers, J.P., Schlesier, B., and Mock, H.P.** (2005). A proteome approach defines protective functions of tobacco leaf trichomes. Proteomics **5,** 2508-2518.

**Assmann, S.M., and Grantz, D.A.** (1990). Stomatal response to humidity in sugarcane and soybean: effect of vapour pressure difference on the kinetics of the blue light response. Plant, Cell and Environ **13,** 163-169.

**Assmann, S.M., and Zeiger, E.** (1987). "Guard cell bioenergetics." In Stomatal Function, E. Zeiger, G. Farquhar, and I. Cowan, eds. (Stanford: Stanford University Press), pp. 163-194.

**Baerenfaller, K., Grossmann, J., Grobei, M.A., Hull, R., Hirsch-Hoffmann, M., Yalovsky, S., Zimmermann, P., Grossniklaus, U., Gruissem, W., and Baginsky, S.** (2008). Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science **320,** 938-941.

**Bailey, B.A., Strem, M.D., Bae, H., de Mayolo, G.A., and Guiltinan, M.J.** (2005). Gene expression in leaves of *Theobroma cacao* in response to mechanical wounding, ethylene, and/or methyl jasmonate. Plant Science **168,** 1247-1258.

**Baldwin, I.T., Halitschke, R., Paschold, A., von Dahl, C.C., and Preston, C.A.** (2006). Volatile signaling in plant-plant interactions: "talking trees" in the genomics era. Science **311,** 812-815.

**Barth, C., and Jander, G.** (2006). Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. Plant J **46,** 549-562.

**Bayer, E.M., Bottrill, A.R., Walshaw, J., Vigouroux, M., Naldrett, M.J., Thomas, C.L., and Maule, A.J.** (2006). Arabidopsis cell wall proteome defined using multidimensional protein identification technology. Proteomics **6,** 301-311.

**Betts, R.A., Boucher, O., Collins, M., Cox, P.M., Falloon, P.D., Gedney, N., Hemming, D.L., Huntingford, C., Jones, C.D., Sexton, D.M., and Webb, M.J.** (2007). Projected increase in continental runoff due to plant responses to increasing carbon dioxide. Nature **448,** 1037-1041.

**Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W., and Benfey, P.N.** (2003). A gene expression map of the Arabidopsis root. Science **302,** 1956-1960.

**Blatt, M.R.** (2000). $Ca^{2+}$ signalling and control of guard-cell volume in stomatal movements. Curr Opin Plant Biol **3,** 196-204.

**Bourgis, F., Botha, F.C., Mani, S., Hiten, F.N., Rigden, D.J., and Verbruggen, N.** (2005). Characterization and functional investigation of an Arabidopsis cDNA encoding a homologue to the d-PGMase superfamily. J Exp Bot **56,** 1129-1142.

**Bown, L., Kusaba, S., Goubet, F., Codrai, L., Dale, A.G., Zhang, Z., Yu, X., Morris, K., Ishii, T., Evered, C., Dupree, P., and Jackson, S.** (2007). The *ectopically parting cells 1-2* (*epc1-2*) mutant exhibits an exaggerated response to abscisic acid. J Exp Bot **58,** 1813-1823.

**Bradburne, R.P., and Mithen, R.** (2000). Glucosinolate genetics and the attraction of the aphid parasitoid *Diaeretiella rapae* to *Brassica*. Proc Biol Sci **267,** 89-95.

**Breshears, D.D., Cobb, N.S., Rich, P.M., Price, K.P., Allen, C.D., Balice, R.G., Romme, W.H., Kastens, J.H., Floyd, M.L., Belnap, J., Anderson, J.J., Myers, O.B., and Meyer, C.W.** (2005). Regional vegetation die-off in response to global-change-type drought. Proc Natl Acad Sci U S A **102,** 15144-15148.

**Bright, J., Desikan, R., Hancock, J.T., Weir, I.S., and Neill, S.J.** (2006). ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on $H_2O_2$ synthesis. Plant J **45,** 113-122.

**Brown, P.D., Tokuhisa, J.G., Reichelt, M., and Gershenzon, J.** (2003). Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry **62,** 471-481.

**Browse, J., McConn, M., James, D., Jr., and Miquel, M.** (1993). Mutants of Arabidopsis deficient in the synthesis of α-linolenate. Biochemical and genetic characterization of the endoplasmic reticulum linoleoyl desaturase. J Biol Chem **268,** 16345-16351.

**Burmeister, W.P., Cottaz, S., Rollin, P., Vasella, A., and Henrissat, B.** (2000). High resolution X-ray crystallography shows that ascorbate is a cofactor for myrosinase and substitutes for the function of the catalytic base. J Biol Chem **275,** 39385-39393.

**Carroll, A.J., Heazlewood, J.L., Ito, J., and Millar, A.H.** (2008). Analysis of the Arabidopsis cytosolic ribosome proteome provides detailed insights into its

components and their post-translational modification. Mol Cell Proteomics **7,** 347-369.

**Chase, C.D.** (2007). Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. Trends in Genetics **23,** 81-90.

**Chen, Z., and Gallie, D.R.** (2004). The ascorbic acid redox state controls guard cell signaling and stomatal movement. Plant Cell **16,** 1143-1162.

**Cheong, Y.H., Pandey, G.K., Grant, J.J., Batistic, O., Li, L., Kim, B.G., Lee, S.C., Kudla, J., and Luan, S.** (2007). Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. Plant J **52,** 223-239.

**Clark, S.E., Jacobsen, S.E., Levin, J.Z., and Meyerowitz, E.M.** (1996). The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in Arabidopsis. Development **122,** 1567-1575.

**Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M., Leonhardt, N., Dellaporta, S.L., and Tonelli, C.** (2005). A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. Curr Biol **15,** 1196-1200.

**Coursol, S., Fan, L.M., Le Stunff, H., Spiegel, S., Gilroy, S., and Assmann, S.M.** (2003). Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. Nature **423,** 651-654.

**Davletova, S., Schlauch, K., Coutu, J., and Mittler, R.** (2005). The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in Arabidopsis. Plant Physiol **139,** 847-856.

**Desbrosses-Fonrouge, A.G., Voigt, K., Schroder, A., Arrivault, S., Thomine, S., and Kramer, U.** (2005). *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. FEBS Lett **579,** 4165-4174.

**Desikan, R., Griffiths, R., Hancock, J., and Neill, S.** (2002). A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **99,** 16314-16318.

**Desikan, R., Hancock, J.T., Bright, J., Harrison, J., Weir, I., Hooley, R., and Neill, S.J.** (2005). A role for ETR1 in hydrogen peroxide signaling in stomatal guard cells. Plant Physiol **137,** 831-834.

**Desikan, R., Last, K., Harrett-Williams, R., Tagliavia, C., Harter, K., Hooley, R., Hancock, J.T., and Neill, S.J.** (2006). Ethylene-induced stomatal closure in Arabidopsis occurs via AtrbohF-mediated hydrogen peroxide synthesis. Plant J **47,** 907-916.

**Desikan, R., Horak, J., Chaban, C., Mira-Rodado, V., Witthoft, J., Elgass, K., Grefen, C., Cheung, M.K., Meixner, A.J., Hooley, R., Neill, S.J., Hancock, J.T., and Harter, K.** (2008). The histidine kinase AHK5 integrates endogenous and environmental signals in Arabidopsis guard cells. PLoS ONE **3,** e2491.

**Diks, S.H., and Peppelenbosch, M.P.** (2004). Single cell proteomics for personalised medicine. Trends Mol Med **10,** 574-577.

**Dinneny, J.R., Long, T.A., Wang, J.Y., Jung, J.W., Mace, D., Pointer, S., Barron, C., Brady, S.M., Schiefelbein, J., and Benfey, P.N.** (2008). Cell identity mediates the response of Arabidopsis roots to abiotic stress. Science **320,** 942-945.

**Dixon, D.P., Skipsey, M., Grundy, N.M., and Edwards, R.** (2005). Stress-induced protein S-glutathionylation in Arabidopsis. Plant Physiol **138,** 2233-2244.

**Emanuelsson, O., Nielsen, H., and von Heijne, G.** (1999). ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci **8,** 978-984.

**Evans, J.R.** (1989). Photosynthesis and nitrogen relationships in leaves of $C_3$ plants. Oecologia **78,** 9-19.

**Fan, L.M., Zhao, Z., and Assmann, S.M.** (2004). Guard cells: a dynamic signaling model. Curr Opin Plant Biol **7,** 537-546.

**Fan, L.M., Zhang, W., Chen, J.G., Taylor, J.P., Jones, A.M., and Assmann, S.M.** (2008a). Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ and RGS–deficient Arabidopsis lines. Proc Natl Acad Sci U S A.

**Fan, L.M., Zhang, W., Chen, J.G., Taylor, J.P., Jones, A.M., and Assmann, S.M.** (2008b). Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ- and RGS-deficient Arabidopsis lines. Proc Natl Acad Sci U S A **105,** 8476-8481.

**Friso, G., Giacomelli, L., Ytterberg, A.J., Peltier, J.B., Rudella, A., Sun, Q., and Wijk, K.J.** (2004). In-depth analysis of the thylakoid membrane proteome of *Arabidopsis thaliana* chloroplasts: new proteins, new functions, and a plastid proteome database. Plant Cell **16,** 478-499.

**Frost, C.J., Appel, H.M., Carlson, J.E., De Moraes, C.M., Mescher, M.C., and Schultz, J.C.** (2007). Within-plant signalling via volatiles overcomes vascular constraints on systemic signalling and primes responses against herbivores. Ecol Lett **10,** 490-498.

**Galbiati, M., Simoni, L., Pavesi, G., Cominelli, E., Francia, P., Vavasseur, A., Nelson, T., Bevan, M., and Tonelli, C.** (2008). Gene trap lines identify Arabidopsis genes expressed in stomatal guard cells. Plant J **53,** 750-762.

**Gobert, A., Isayenkov, S., Voelker, C., Czempinski, K., and Maathuis, F.J.** (2007). The two-pore channel *TPK1* gene encodes the vacuolar $K^+$ conductance and plays a role in $K^+$ homeostasis. Proc Natl Acad Sci U S A **104,** 10726-10731.

**Gotow, K., Taylor, S., and Zeiger, E.** (1988). Photosynthetic carbon fixation in guard cell protoplasts of *Vicia faba* L. : evidence from radiolabel experiments. Plant Physiol **86,** 700-705.

**Grana, X., Perez de la Ossa, P., Broceno, C., Stocker, M., Garriga, J., Puigdomenech, P., and Climent, F.** (1995). 2,3-Bisphosphoglycerate-independent phosphoglycerate mutase is conserved among different phylogenic kingdoms. Comp Biochem Physiol B Biochem Mol Biol **112,** 287-293.

**Gray, J.E., Holroyd, G.H., van der Lee, F.M., Bahrami, A.R., Sijmons, P.C., Woodward, F.I., Schuch, W., and Hetherington, A.M.** (2000). The HIC signalling pathway links $CO_2$ perception to stomatal development. Nature **408,** 713-716.

**Grubb, C.D., and Abel, S.** (2006). Glucosinolate metabolism and its control. Trends Plant Sci **11,** 89-100.

**Guo, F.Q., Young, J., and Crawford, N.M.** (2003). The nitrate transporter AtNRT1.1 (CHL1) functions in stomatal opening and contributes to drought susceptibility in Arabidopsis. Plant Cell **15,** 107-117.

**Halkier, B.A., and Gershenzon, J.** (2006). Biology and biochemistry of glucosinolates. Ann Rev of Plant Bio **57,** 303-333.

**Han, S., Tang, R., Anderson, L.K., Woerner, T.E., and Pei, Z.M.** (2003). A cell surface receptor mediates extracellular $Ca^{2+}$ sensing in guard cells. Nature **425,** 196-200.

**Hansen, B.G., Kliebenstein, D.J., and Halkier, B.A.** (2007). Identification of a flavin-monooxygenase as the S-oxygenating enzyme in aliphatic glucosinolate biosynthesis in Arabidopsis. Plant J **50,** 902-910.

**Hashimoto, M., and Komatsu, S.** (2007). Proteomic analysis of rice seedlings during cold stress. Proteomics **7,** 1293-1302.

**Hashimoto, M., Negi, J., Young, J., Israelsson, M., Schroeder, J.I., and Iba, K.** (2006). Arabidopsis HT1 kinase controls stomatal movements in response to $CO_2$. Nat Cell Biol **8,** 391-397.

**Heazlewood, J.L., Tonti-Filippini, J.S., Gout, A.M., Day, D.A., Whelan, J., and Millar, A.H.** (2004). Experimental analysis of the Arabidopsis mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell **16,** 241-256.

**Hedrich, R., Raschke, K., and Stitt, M.** (1985). A role for fructose 2,6-bisphosphate in regulating carbohydrate metabolism in guard cells. Plant Physiol **79,** 977-982.

**Hetherington, A.M., and Woodward, F.I.** (2003). The role of stomata in sensing and driving environmental change. Nature **424,** 901-908.

**Hinman, A., Chuang, H.H., Bautista, D.M., and Julius, D.** (2006). TRP channel activation by reversible covalent modification. Proc Natl Acad Sci U S A **103,** 19564-19568.

**Holmes-Davis, R., Tanaka, C.K., Vensel, W.H., Hurkman, W.J., and McCormick, S.** (2005). Proteome mapping of mature pollen of Arabidopsis thaliana. Proteomics **5,** 4864-4884.

**Hosy, E., Vavasseur, A., Mouline, K., Dreyer, I., Gaymard, F., Poree, F., Boucherez, J., Lebaudy, A., Bouchez, D., Very, A.A., Simonneau, T., Thibaud, J.B., and Sentenac, H.** (2003). The *Arabidopsis* outward $K^+$ channel GORK is involved in regulation of stomatal movements and plant transpiration. Proc Natl Acad Sci U S A **100,** 5549-5554.

**Hugouvieux, V., Kwak, J.M., and Schroeder, J.I.** (2001). An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis. Cell **106,** 477-487.

**Husebye, H., Chadchawan, S., Winge, P., Thangstad, O.P., and Bones, A.M.** (2002). Guard cell- and phloem idioblast-specific expression of thioglucoside glucohydrolase 1 (myrosinase) in Arabidopsis. Plant Physiol **128,** 1180-1188.

**Israelsson, M., Siegel, R.S., Young, J., Hashimoto, M., Iba, K., and Schroeder, J.I.** (2006). Guard cell ABA and $CO_2$ signaling network updates and $Ca^{2+}$ sensor priming hypothesis. Curr Opin Plant Biol **9,** 654-663.

**Ivashikina, N., Deeken, R., Fischer, S., Ache, P., and Hedrich, R.** (2005). AKT2/3 subunits render guard cell $K^+$ channels $Ca^{2+}$ sensitive. J Gen Physiol **125,** 483-492.

**Jedrzejas, M.J.** (2000). Structure, function, and evolution of phosphoglycerate mutases: comparison with fructose-2,6-bisphosphatase, acid phosphatase, and alkaline phosphatase. Prog Biophys Mol Biol **73,** 263-287.

**Jeon, B.W., Hwang, J.U., Hwang, Y., Song, W.Y., Fu, Y., Gu, Y., Bao, F., Cho, D., Kwak, J.M., Yang, Z., and Lee, Y.** (2008). The Arabidopsis small G protein ROP2 is activated by light in guard cells and inhibits light-induced stomatal opening. Plant Cell **20,** 75-87.

**Job, C., Rajjou, L., Lovigny, Y., Belghazi, M., and Job, D.** (2005). Patterns of protein oxidation in Arabidopsis seeds and during germination. Plant Physiol **138,** 790-802.

**Johnson, I.T.** (2002). Glucosinolates in the human diet. Bioavailability and implications for health. Phytochemistry Reviews **1,** 183-188.

**Johnson, M.A., von Besser, K., Zhou, Q., Smith, E., Aux, G., Patton, D., Levin, J.Z., and Preuss, D.** (2004). Arabidopsis hapless mutations define essential gametophytic functions. Genetics **168,** 971-982.

**Jung, C., Seo, J.S., Han, S.W., Koo, Y.J., Kim, C.H., Song, S.I., Nahm, B.H., Choi, Y.D., and Cheong, J.J.** (2008). Overexpression of *AtMYB44* enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. Plant Physiol **146,** 623-635.

**Kim, J.S., Jung, H.J., Lee, H.J., Kim, K.A., Goh, C.H., Woo, Y., Oh, S.H., Han, Y.S., and Kang, H.** (2008). Glycine-rich RNA-binding protein7 affects abiotic stress responses by regulating stomata opening and closing in *Arabidopsis thaliana*. Plant J.

**Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M., and Shimazaki, K.** (2001). Phot1 and phot2 mediate blue light regulation of stomatal opening. Nature **414,** 656-660.

**Kleffmann, T., von Zychlinski, A., Russenberger, D., Hirsch-Hoffmann, M., Gehrig, P., Gruissem, W., and Baginsky, S.** (2007). Proteome dynamics during plastid differentiation in rice. Plant Physiol **143,** 912-923.

**Klein, M., Perfus-Barbeoch, L., Frelet, A., Gaedeke, N., Reinhardt, D., Mueller-Roeber, B., Martinoia, E., and Forestier, C.** (2003). The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signalling and water use. Plant J **33,** 119-129.

**Klein, M., Geisler, M., Suh, S.J., Kolukisaoglu, H.U., Azevedo, L., Plaza, S., Curtis, M.D., Richter, A., Weder, B., Schulz, B., and Martinoia, E.** (2004). Disruption of AtMRP4, a guard cell plasma membrane ABCC-type ABC transporter, leads to deregulation of stomatal opening and increased drought susceptibility. Plant J **39,** 219-236.

**Kwak, J.M., Moon, J.H., Murata, Y., Kuchitsu, K., Leonhardt, N., DeLong, A., and Schroeder, J.I.** (2002). Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, RCN1, confers abscisic acid insensitivity in Arabidopsis. Plant Cell **14,** 2849-2861.

**Kwak, J.M., Murata, Y., Baizabal-Aguirre, V.M., Merrill, J., Wang, M., Kemper, A., Hawke, S.D., Tallman, G., and Schroeder, J.I.** (2001). Dominant negative guard cell $K^+$ channel mutants reduce inward-rectifying $K^+$ currents and light-induced stomatal opening in arabidopsis. Plant Physiol **127,** 473-485.

**Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., and Schroeder, J.I.** (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis. Embo J **22,** 2623-2633.

**Kwon, S.J., Choi, E.Y., Choi, Y.J., Ahn, J.H., and Park, O.K.** (2006). Proteomics studies of post-translational modifications in plants. J Exp Bot **57,** 1547-1551.

**Lambert, J.P., Ethier, M., Smith, J.C., and Figeys, D.** (2005). Proteomics: from gel based to gel free. Anal. Chem. **77,** 3771-3788.

**Lasceve, G., Leymarie, J., and Vavasseur, A.** (1997). Alterations in light-induced stomatal opening in a starch-deficient mutant of *Arabidopsis thaliana* L deficient in chloroplast phosphoglucomutase activity. Plant Cell and Envir **20,** 350-358.

**Lebaudy, A., Vavasseur, A., Hosy, E., Dreyer, I., Leonhardt, N., Thibaud, J.B., Very, A.A., Simonneau, T., and Sentenac, H.** (2008). Plant adaptation to fluctuating environment and biomass production are strongly dependent on guard cell potassium channels. Proc Natl Acad Sci U S A **105,** 5271-5276.

**Lee, J., Garrett, W.M., and Cooper, B.** (2007a). Shotgun proteomic analysis of *Arabidopsis thaliana* leaves. J Sep Sci **30,** 2225-2230.

**Lee, S.-J., Saravanan, R.S., Damasceno, C.M.B., Yamane, H., Kim, B.-D., and Rose, J.K.C.** (2004). Digging deeper into the plant cell wall proteome. Plant Physiol and Biochemistry **42,** 979-988.

**Lee, Y., Kim, Y.W., Jeon, B.W., Park, K.Y., Suh, S.J., Seo, J., Kwak, J.M., Martinoia, E., Hwang, I., and Lee, Y.** (2007b). Phosphatidylinositol 4,5-bisphosphate is important for stomatal opening. Plant J **52,** 803-816.

**Lemichez, E., Wu, Y., Sanchez, J.-P., Mettouchi, A., Mathur, J., and Chua, N.-H.** (2001). Inactivation of AtRac1 by abscisic acid is essential for stomatal closure. Genes & Dev. **15,** 1808-1816.

**Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G., and Schroeder, J.I.** (2004). Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell **16,** 596-615.

**Li, J., Wang, X.Q., Watson, M.B., and Assmann, S.M.** (2000). Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science **287,** 300-303.

**Li, S., Assmann, S.M., and Albert, R.** (2006). Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. PLoS Biol **4,** e312.

**Liang, Y.K., Dubos, C., Dodd, I.C., Holroyd, G.H., Hetherington, A.M., and Campbell, M.M.** (2005). AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. Curr Biol **15,** 1201-1206.

**Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W.H., and Ma, L.** (2007). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science **315,** 1712-1716.

**Maffei, M.E., Mithofer, A., and Boland, W.** (2007). Before gene expression: early events in plant-insect interaction. Trends Plant Sci **12,** 310-316.

**March-Diaz, R., Garcia-Dominguez, M., Lozano-Juste, J., Leon, J., Florencio, F.J., and Reyes, J.C.** (2008). Histone H2A.Z and homologues of components of the SWR1 complex are required to control immunity in Arabidopsis. Plant J **53,** 475-487.

**Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S.Y.** (2006). Plant stomata function in innate immunity against bacterial invasion. Cell **126,** 969-980.

**Melquist, S., and Bender, J.** (2004). An internal rearrangement in an Arabidopsis inverted repeat locus impairs DNA methylation triggered by the locus. Genetics **166,** 437-448.

**Merlot, S., Leonhardt, N., Fenzi, F., Valon, C., Costa, M., Piette, L., Vavasseur, A., Genty, B., Boivin, K., Muller, A., Giraudat, J., and Leung, J.** (2007). Constitutive activation of a plasma membrane $H^+$-ATPase prevents abscisic acid-mediated stomatal closure. Embo J **26,** 3216-3226.

**Miao, Y., Lv, D., Wang, P., Wang, X.C., Chen, J., Miao, C., and Song, C.P.** (2006). An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Cell **18,** 2749-2766.

**Mishra, G., Zhang, W., Deng, F., Zhao, J., and Wang, X.** (2006). A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science **312,** 264-266.

**Mitchum, M.G., Wang, X., and Davis, E.L.** (2008). Diverse and conserved roles of CLE peptides. Curr Opin Plant Biol **11,** 75-81.

**Mori, I.C., Murata, Y., Yang, Y., Munemasa, S., Wang, Y.F., Andreoli, S., Tiriac, H., Alonso, J.M., Harper, J.F., Ecker, J.R., Kwak, J.M., and Schroeder, J.I.** (2006). CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and $Ca^{2+}$-permeable channels and stomatal closure. PLoS Biol **4,** e327.

**Munemasa, S., Oda, K., Watanabe-Sugimoto, M., Nakamura, Y., Shimoishi, Y., and Murata, Y.** (2007). The coronatine-insensitive 1 mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in Arabidopsis guard cells. Specific impairment of ion channel activation and second messenger production. Plant Physiol **143,** 1398-1407.

**Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J.** (2002). Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell **14,** 3089-3099.

**Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., Hashimoto, M., and Iba, K.** (2008). $CO_2$ regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature **452,** 483-486.

**Noir, S., Brautigam, A., Colby, T., Schmidt, J., and Panstruga, R.** (2005). A reference map of the *Arabidopsis thaliana* mature pollen proteome. Biochem Biophys Res Commun **337,** 1257-1266.

**Osakabe, Y., Maruyama, K., Seki, M., Satou, M., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2005). Leucine-rich repeat receptor-like kinase1 is a key membrane-bound regulator of abscisic acid early signaling in Arabidopsis. Plant Cell **17,** 1105-1119.

**Padmanaban, S., Chanroj, S., Kwak, J.M., Li, X., Ward, J.M., and Sze, H.** (2007). Participation of endomembrane cation/$H^+$ exchanger AtCHX20 in osmoregulation of guard cells. Plant Physiol **144,** 82-93.

**Pandey, S., and Assmann, S.M.** (2004). The Arabidopsis putative G protein-coupled receptor GCR1 interacts with the G protein $\alpha$ subunit GPA1 and regulates abscisic acid signaling. Plant Cell **16,** 1616-1632.

**Pandey, S., Zhang, W., and Assmann, S.M.** (2007). Roles of ion channels and transporters in guard cell signal transduction. FEBS Lett **581,** 2325-2336.

**Pandey, S., Wang, X.Q., Coursol, S.A., and Assmann, S.M.** (2002). Preparation and applications of *Arabidopsis thaliana* guard cell protoplasts. New Phytologist **153,** 517.

**Parrish, J.R., Yu, J., Liu, G., Hines, J.A., Chan, J.E., Mangiola, B.A., Zhang, H., Pacifico, S., Fotouhi, F., DiRita, V.J., Ideker, T., Andrews, P., and Finley, R.L., Jr.** (2007). A proteome-wide protein interaction map for *Campylobacter jejuni*. Genome Biol **8,** R130.

**Pei, Z.M., Ghassemian, M., Kwak, C.M., McCourt, P., and Schroeder, J.I.** (1998). Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science **282,** 287-290.

**Peiter, E., Maathuis, F.J., Mills, L.N., Knight, H., Pelloux, J., Hetherington, A.M., and Sanders, D.** (2005). The vacuolar $Ca^{2+}$-activated channel TPC1 regulates germination and stomatal movement. Nature **434,** 404-408.

**Petersen, B., Chen, S., Hansen, C., Olsen, C., and Halkier, B.** (2002). Composition and content of glucosinolates in developing *Arabidopsis thaliana*. Planta **214,** 562-571.

**Philippar, K., Geis, T., Ilkavets, I., Oster, U., Schwenkert, S., Meurer, J., and Soll, J.** (2007). Chloroplast biogenesis: the use of mutants to study the etioplast-chloroplast transition. Proc Natl Acad Sci U S A **104,** 678-683.

**Pilot, G., Lacombe, B., Gaymard, F., Cherel, I., Boucherez, J., Thibaud, J.B., and Sentenac, H.** (2001). Guard cell inward $K^+$ channel activity in arabidopsis involves expression of the twin channel subunits KAT1 and KAT2. J Biol Chem **276,** 3215-3221.

**Plaxton, W.C.** (1996). The organization and regulation of plant glycolysis. Annu Rev Plant Physiol Plant Mol Biol **47,** 185-214.

**Popova, L.P., Tsonev, T.D., and Vaklinova, S.G.** (1987). A possible role for abscisic acid in regulation of photosynthetic and photorespiratory carbon metabolism in barley leaves. Plant Physiol **83,** 820-824.

**Quettier, A.L., Bertrand, C., Habricot, Y., Miginiac, E., Agnes, C., Jeannette, E., and Maldiney, R.** (2006). The *phs1-3* mutation in a putative dual-specificity

protein tyrosine phosphatase gene provokes hypersensitive responses to abscisic acid in *Arabidopsis thaliana*. Plant J **47,** 711-719.

**Rajjou, L., Gallardo, K., Debeaujon, I., Vandekerckhove, J., Job, C., and Job, D.** (2004). The effect of α-amanitin on the Arabidopsis seed proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination. Plant Physiol **134,** 1598-1613.

**Rossignol, M., Peltier, J.B., Mock, H.P., Matros, A., Maldonado, A.M., and Jorrin, J.V.** (2006). Plant proteome analysis: a 2004-2006 update. Proteomics **6,** 5529-5548.

**Saez, A., Robert, N., Maktabi, M.H., Schroeder, J.I., Serrano, R., and Rodriguez, P.L.** (2006). Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. Plant Physiol **141,** 1389-1399.

**Schirle, M., Heurtier, M.A., and Kuster, B.** (2003). Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. Mol Cell Proteomics **2,** 1297-1305.

**Schmelz, E.A., Engelberth, J., Alborn, H.T., O'Donnell, P., Sammons, M., Toshima, H., and Tumlinson, J.H., 3rd.** (2003). Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. Proc Natl Acad Sci U S A **100,** 10552-10557.

**Schroter, D., Cramer, W., Leemans, R., Prentice, I.C., Araujo, M.B., Arnell, N.W., Bondeau, A., Bugmann, H., Carter, T.R., Gracia, C.A., de la Vega-Leinert, A.C., Erhard, M., Ewert, F., Glendining, M., House, J.I., Kankaanpaa, S., Klein, R.J., Lavorel, S., Lindner, M., Metzger, M.J., Meyer, J., Mitchell, T.D., Reginster, I., Rounsevell, M., Sabate, S., Sitch, S., Smith, B., Smith, J., Smith, P., Sykes, M.T., Thonicke, K., Thuiller, W., Tuck, G., Zaehle, S., and Zierl, B.** (2005). Ecosystem service supply and vulnerability to global change in Europe. Science **310,** 1333-1337.

**Schwartz, A., and Zeiger, E.** (1984). Metabolic energy for stomatal opening. Roles of photophosphorylation and oxidative phosphorylation. Planta **161,** 129-136.

**Shen, Y.Y., Wang, X.F., Wu, F.Q., Du, S.Y., Cao, Z., Shang, Y., Wang, X.L., Peng, C.C., Yu, X.C., Zhu, S.Y., Fan, R.C., Xu, Y.H., and Zhang, D.P.** (2006). The Mg-chelatase H subunit is an abscisic acid receptor. Nature **443,** 823-826.

**Shilov, I.V., Seymour, S.L., Patel, A.A., Loboda, A., Tang, W.H., Keating, S.P., Hunter, C.L., Nuwaysir, L.M., and Schaeffer, D.A.** (2007). The Paragon algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics **6,** 1638-1655.

**Shimazaki, K., Doi, M., Assmann, S.M., and Kinoshita, T.** (2007). Light regulation of stomatal movement. Annu Rev Plant Biol **58,** 219-247.

**Shimazaki, K.I.** (1989). Ribulosebisphosphate carboxylase activity and photosynthetic $O_2$ evolution rate in *Vicia* guard-cell protoplasts. Plant Physiol **91,** 459-463.

**Song, C.P., Agarwal, M., Ohta, M., Guo, Y., Halfter, U., Wang, P., and Zhu, J.K.** (2005). Role of an Arabidopsis AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. Plant Cell **17,** 2384-2396.

**Sorin, C., Negroni, L., Balliau, T., Corti, H., Jacquemot, M.P., Davanture, M., Sandberg, G., Zivy, M., and Bellini, C.** (2006). Proteomic analysis of different mutant genotypes of Arabidopsis led to the identification of 11 proteins correlating with adventitious root development. Plant Physiol **140,** 349-364.

**Stadler, R., Buttner, M., Ache, P., Hedrich, R., Ivashikina, N., Melzer, M., Shearson, S.M., Smith, S.M., and Sauer, N.** (2003). Diurnal and light-regulated expression of AtSTP1 in guard cells of Arabidopsis. Plant Physiol **133,** 528-537.

**Suhita, D., Raghavendra, A.S., Kwak, J.M., and Vavasseur, A.** (2004). Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. Plant Physiol **134,** 1536-1545.

**Svennerstam, H., Ganeteg, U., Bellini, C., and Nasholm, T.** (2007). Comprehensive screening of Arabidopsis mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids. Plant Physiol **143,** 1853-1860.

**Tabb, D.L.** (2008). What's driving false discovery rates? J Proteome Res **7,** 45-46.

**Tyers, M., and Mann, M.** (2003). From genomics to proteomics. Nature **422,** 193-197.

**Vavasseur, A., and Raghavendra, A.S.** (2005). Guard cell metabolism and $CO_2$ sensing. New Phytol **165,** 665-682.

**Wall, M.K., Mitchenall, L.A., and Maxwell, A.** (2004). *Arabidopsis thaliana* DNA gyrase is targeted to chloroplasts and mitochondria. Proc Natl Acad Sci U S A **101,** 7821-7826.

**Wan, J., Torres, M., Ganapathy, A., Thelen, J., DaGue, B.B., Mooney, B., Xu, D., and Stacey, G.** (2005). Proteomic analysis of soybean root hairs after infection by *Bradyrhizobium japonicum*. Mol Plant Microbe Interact **18,** 458-467.

**Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M.** (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science **292,** 2070-2072.

**Welchen, E., and Gonzalez, D.H.** (2006). Overrepresentation of elements recognized by TCP-domain transcription factors in the upstream regions of nuclear genes encoding components of the mitochondrial oxidative phosphorylation Machinery. Plant Physiol **141,** 540-545.

**Westram, A., Lloyd, J.R., Roessner, U., Riesmeier, J.W., and Kossmann, J.** (2002). Increases of 3-phosphoglyceric acid in potato plants through antisense reduction of cytoplasmic phosphoglycerate mutase impairs photosynthesis and growth, but does not increase starch contents. Plant, Cell & Environment **25,** 1133-1143.

**Weyers, J.D.B., Paterson, N.W., Fitzsimons, P.J., and Dudley, J.M.** (1982). Metabolic inhibitors block ABA-induced stomatal closure. J. Exp. Bot. **33,** 1270-1278.

**Wienkoop, S., Zoeller, D., Ebert, B., Simon-Rosin, U., Fisahn, J., Glinski, M., and Weckwerth, W.** (2004). Cell-specific protein profiling in *Arabidopsis thaliana* trichomes: identification of trichome-located proteins involved in sulfur metabolism and detoxification. Phytochemistry **65,** 1641-1649.

**Wittstock, U., and Halkier, B.A.** (2002). Glucosinolate research in the Arabidopsis era. Trends Plant Sci **7,** 263-270.

**Xie, X., Wang, Y., Williamson, L., Holroyd, G.H., Tagliavia, C., Murchie, E., Theobald, J., Knight, M.R., Davies, W.J., Leyser, H.M.O., and Hetherington,**

**A.M.** (2006). The identification of genes involved in the stomatal response to reduced atmospheric relative humidity. Current Biol **16,** 882-887.

**Xing, Y., Jia, W., and Zhang, J.** (2007). AtMEK1 mediates stress-induced gene expression of CAT1 catalase by triggering $H_2O_2$ production in Arabidopsis. J Exp Bot **58,** 2969-2981.

**Yan, X., and Chen, S.** (2007). Regulation of plant glucosinolate metabolism. Planta **226,** 1343-1352.

**Yang, Y., Costa, A., Leonhardt, N., Siegel, R.S., and Schroeder, J.I.** (2008). Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. Plant Methods **4,** 6.

**Zhang, W., Qin, C., Zhao, J., and Wang, X.** (2004). Phospholipase D alpha 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci U S A **101,** 9508-9513.

**Zhang, Y., Yang, C., Li, Y., Zheng, N., Chen, H., Zhao, Q., Gao, T., Guo, H., and Xie, Q.** (2007). SDIR1 is a RING finger E3 ligase that positively regulates stress-responsive abscisic acid signaling in Arabidopsis. Plant Cell **19,** 1912-1929.

**Zheng, Z.L., Nafisi, M., Tam, A., Li, H., Crowell, D.N., Chary, S.N., Schroeder, J.I., Shen, J., and Yang, Z.** (2002). Plasma membrane-associated ROP10 small GTPase is a specific negative regulator of abscisic acid responses in Arabidopsis. Plant Cell **14,** 2787-2797.

**Zhu, J., Gong, Z., Zhang, C., Song, C.P., Damsz, B., Inan, G., Koiwa, H., Zhu, J.K., Hasegawa, P.M., and Bressan, R.A.** (2002). OSM1/SYP61: a syntaxin protein in Arabidopsis controls abscisic acid-mediated and non-abscisic acid-mediated responses to abiotic stress. Plant Cell **14,** 3009-3028.

**Zhu, S.Y., Yu, X.C., Wang, X.J., Zhao, R., Li, Y., Fan, R.C., Shang, Y., Du, S.Y., Wang, X.F., Wu, F.Q., Xu, Y.H., Zhang, X.Y., and Zhang, D.P.** (2007). Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. Plant Cell **19,** 3019-3036.

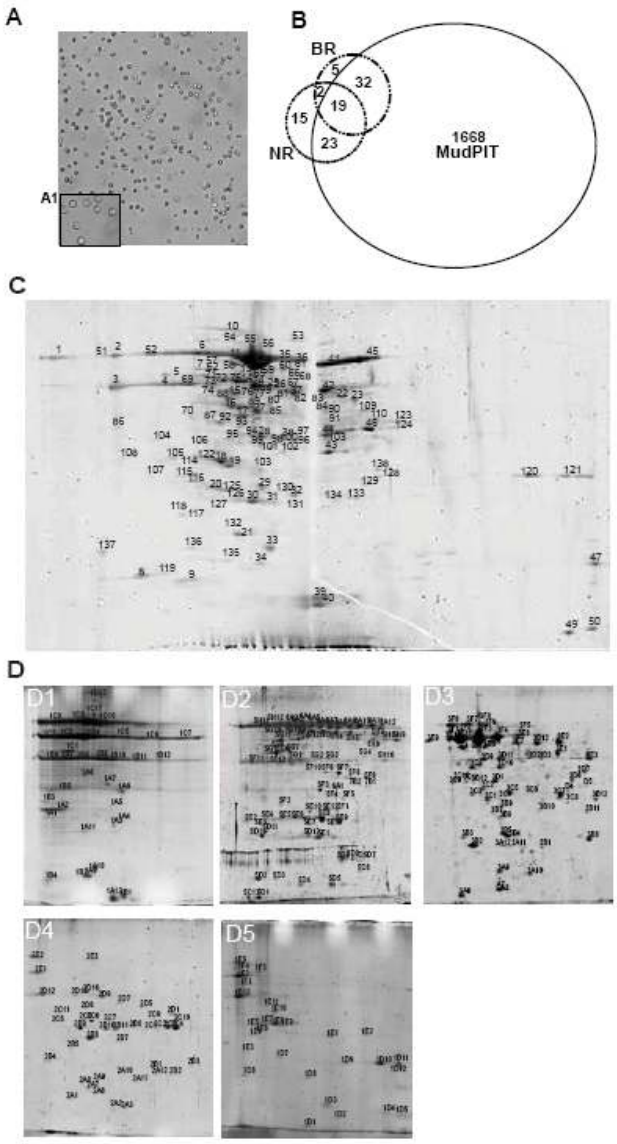



**Figure 2-1. Three proteomic methods identified 1,764 unique proteins of the Arabidopsis guard cell proteome.**

(A) Images showing high purity GCP preparations (100x; insert magnification 400x).
(B) Fifty-eight, 59 and 1,742 proteins were identified from BR, NR and 2D LC-MALDI MudPIT methods respectively; 19 proteins were identified by all three methods. For each method, two independent biological samples were analyzed.
(C) A 2D gel image from the broad pH range method. The first dimension was run using a 24 cm, pH 3-10 IPG strip. In total, 138 protein spots were detected via Coomassie Brilliant Blue G-250 staining.
(D) 2D gel images from the narrow pH range method. Proteins were first fractionated into 5 fractions and each protein fraction was separated on a narrow pH range IPG strip. From B1-B5, the pH ranges are 3-6, 4.5-5.5, 5.3-6.3, 6.1-7.1, 6-10 respectively.

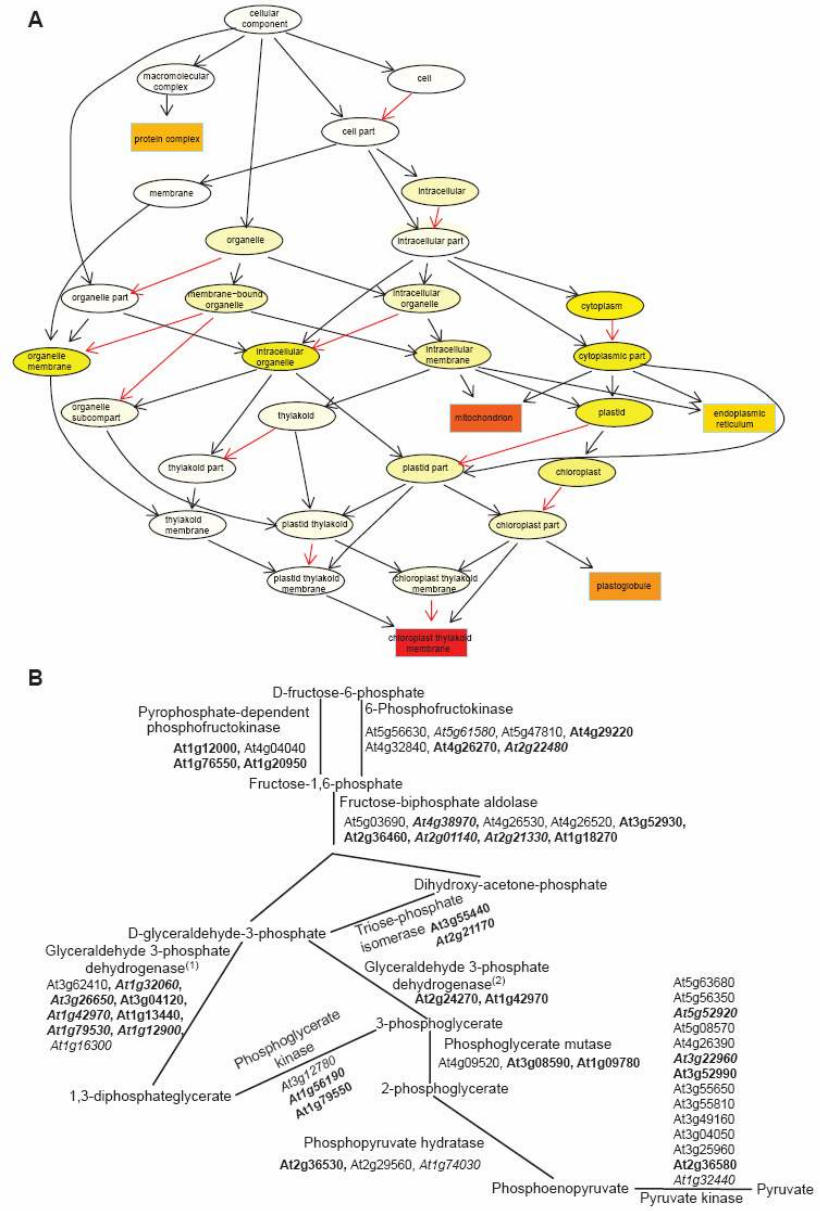



**Figure 2-1. Proteins involved in energy provision are enriched in the identified guard cell proteome.**

(A) Proteins localized in chloroplast thylakoid membranes and mitochondria are enriched in our guard cell proteome. This figure contains the top five GO terms found using the topGO package (Alexa et al., 2006) for Cellular Component. Dark red (P=1E-30), light yellow (P=1E-20), white (non-significant). Black arrow indicates "is a relationship", red arrow indicates "is part of a relationship".
(B) Identified guard cell proteins can cover all steps in glycolysis. All glycolysis proteins are depicted in panel (B). Proteins in boldface were detected in our guard cell proteome. Proteins in italics are predicted to have chloroplast transit peptides by ChloroP (http://www.cbs.dtu.dk/services/ChloroP/). (1) and (2) indicate phosphorylating and non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase respectively.

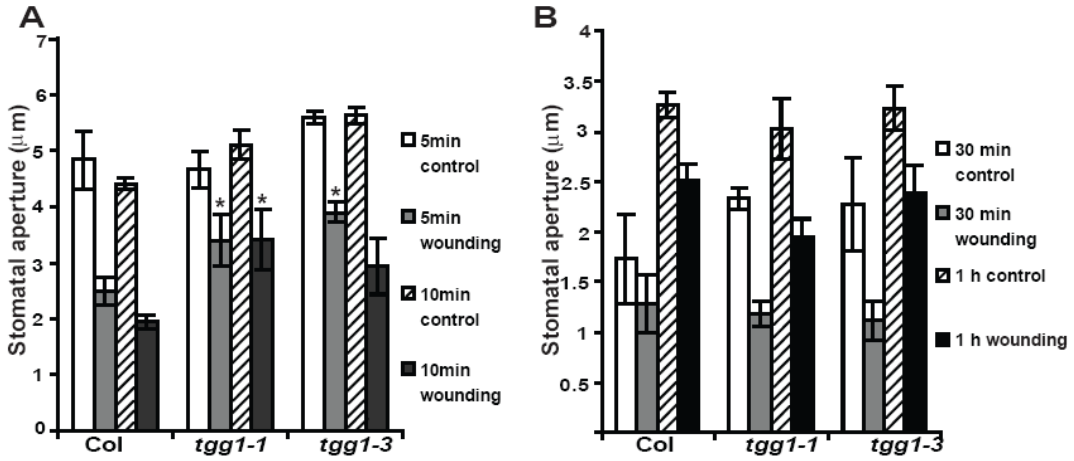
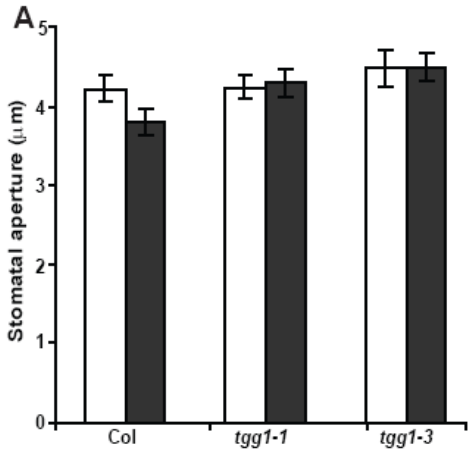
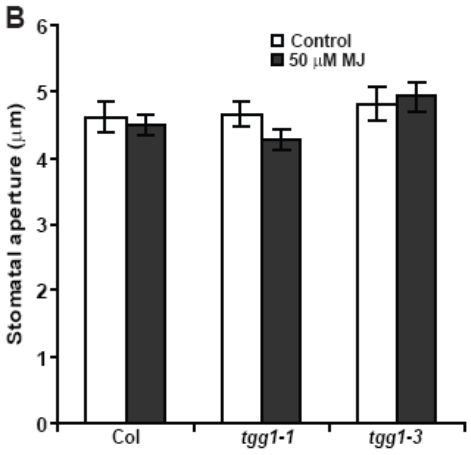



**Figure 2-2. *tgg1* mutants are hyposensitive to wounding promotion of stomatal closure at 5 min.**

We discovered that wounding induces stomatal closure. Stomata in *tgg1* mutants closed more slowly at 5 min after mechanical wounding as compared to wild type, indicating that the glucosinoate-myrosinase system regulates wound-induced stomatal movement
(A) At 5 min, *tgg1-1* and *tgg1-3* mutants are hyposensitive to wounding promotion of stomatal closure as compared to Col (B) *tgg1-1* and *tgg1-3* mutants show no significant difference compared to Col in wounding inhibition of stomatal opening at 30 min and 1 hr.
For (A) and (B), leaves were treated blindly and simultaneously with a slicker brush (Bailey et al., 2005) to ensure the same amounts of tiny holes were punched in all leaves. n = 4 independent experiments. At least 60 stomata were measured for each treatment per genotype per replicate. Data are represented as mean ± SE. Data were analyzed by Student's t-test, $P < 0.05$ was considered significant.




| R | Col | *tgg1-1* | *tgg1-3* |
|---|---|---|---|
| 1 | 0.85 | 1.07 | 0.97 |
| 2 | 0.80 | 0.96 | 1.07 |
| 3 | 0.97 | 1.04 | 0.92 |
| 4 | 0.80 | 0.93 | 0.81 |
| 5 | 0.78 | 0.94 | 1.24 |
| 6 | 0.93 | 1.01 | 0.77 |
| 7 | 1.22 | 1.04 | 1.21 |
| 8 | 0.93 | 1.12 | 1.20 |

| R | Col | *tgg1-1* | *tgg1-3* |
|---|---|---|---|
| 1 | 1.11 | 0.99 | 1.16 |
| 2 | 1.04 | 0.94 | 0.95 |
| 3 | 1.12 | 0.96 | 1.33 |
| 4 | 0.95 | 0.95 | 1.44 |
| 5 | 0.85 | 0.91 | 0.96 |
| 6 | 1.22 | 0.83 | 0.93 |
| 7 | 0.94 | 0.95 | 0.97 |
| 8 | 0.84 | 0.91 | 1.01 |
| 9 | 0.90 | 0.76 | 0.78 |
| 10 | 0.95 | 1.01 | 0.89 |

**Figure 2-3**. **MJ does not affect stomatal movements.**

Wounding induces methyl jasmonate (MJ) accumulation (Maffei et al., 2007), and MJ has been reported to promote stomatal closure (Suhita et al., 2004). We evaluated MJ regulation of stomatal movements in Col and *tgg1* mutants, however, no consistent results were obtained (Figure S5).
(A) MJ does not inhibit stomatal opening in Col or in *tgg1* mutants.
(B) MJ does not promote stomatal closure in Col or in *tgg1* mutants.
n = 8 or 10 independent experiments for opening or closure experiment respectively, with at least 60 stomata measured for each treatment per genotype per replicate. Ratios from each individual replicate are listed in the table below the figure. The ratio = stomatal aperture with MJ treatment /stomatal aperture without MJ treatment.

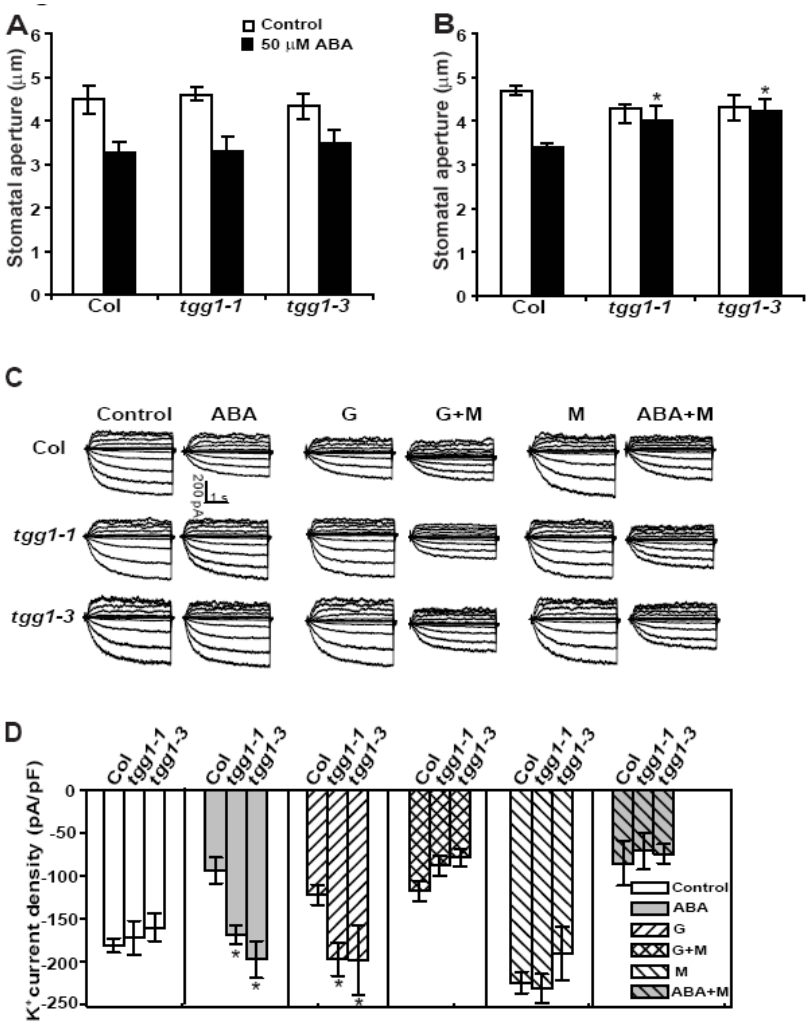



**Figure 2-4**. **Regulatory effects of glucosinolates and/or myrosinases on Arabidopsis stomatal apertures and guard cell $K^+_{in}$ currents of Col and *tgg1* mutants in the presence and absence of ABA.**

(A) *tgg1-1* and *tgg1-3* mutants respond similarly to wild-type (Col) in ABA induction of stomatal closure.
(B) *tgg1-1* and *tgg1-3* mutants are hyposensitive to ABA-inhibition of stomatal opening.
For (A) and (B), n = 3 experiments. Data are mean ± SE. $P < 0.05$ (Student's t-test) was considered significant (*).
(C) Typical guard cell whole-cell $K^+$ current traces of Col and *tgg1* mutants under control, 50 μM glucosinolate (G) and/or 0.2 unit/ml myrosinase (M) treatments, with or without 50 μM ABA. Current and time scales are as shown.
(D) $K^+$ current density (mean ± SE) at -219 mV of guard cell whole-cell inward $K^+$ currents.
G = glucosinolates, M = myrosinase. N = 14, 7, 6, 10, 7, 6 for Col under control, ABA, G, G+M, M, ABA+M treatments respectively; n = 13, 10, 11, 6, 7, 7 for *tgg1-1* under control, ABA, G, G+M, M, ABA+M treatments respectively; n = 16, 8, 13, 8, 6, 12 for *tgg1-3* under control, ABA, G, G+M, M, ABA+M treatments respectively. $P \leq 0.01$ (Student's t-test) was considered significant (*) for D.

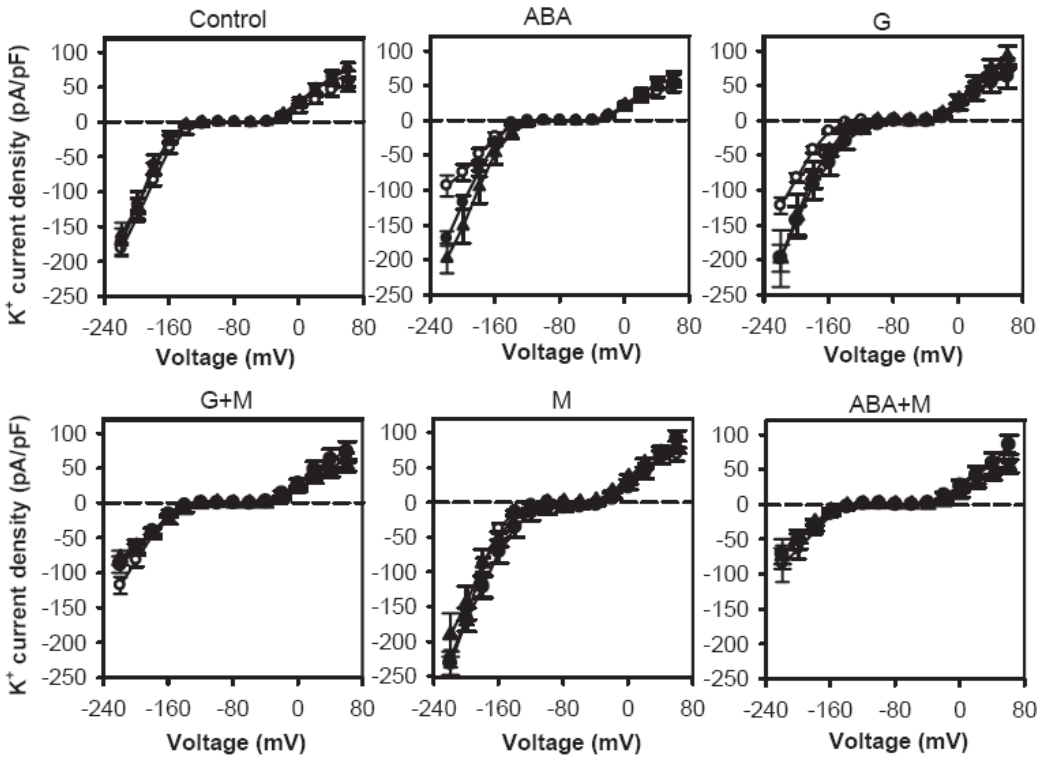



**Figure 2-5. I/V curves of time-activated whole-cell $K^+$ currents of Col and *tgg1* mutant guard cells of Figure 3C.**

Current amplitudes (mean ± SE) at different voltages were obtained by subtracting the instantaneous current at 20 ms from the average steady-state current between 3.55 and 3.87 s. Cells used for these calculations are the same as those for Figure 3D in the main text, and identical symbols are used to designate genotypes.

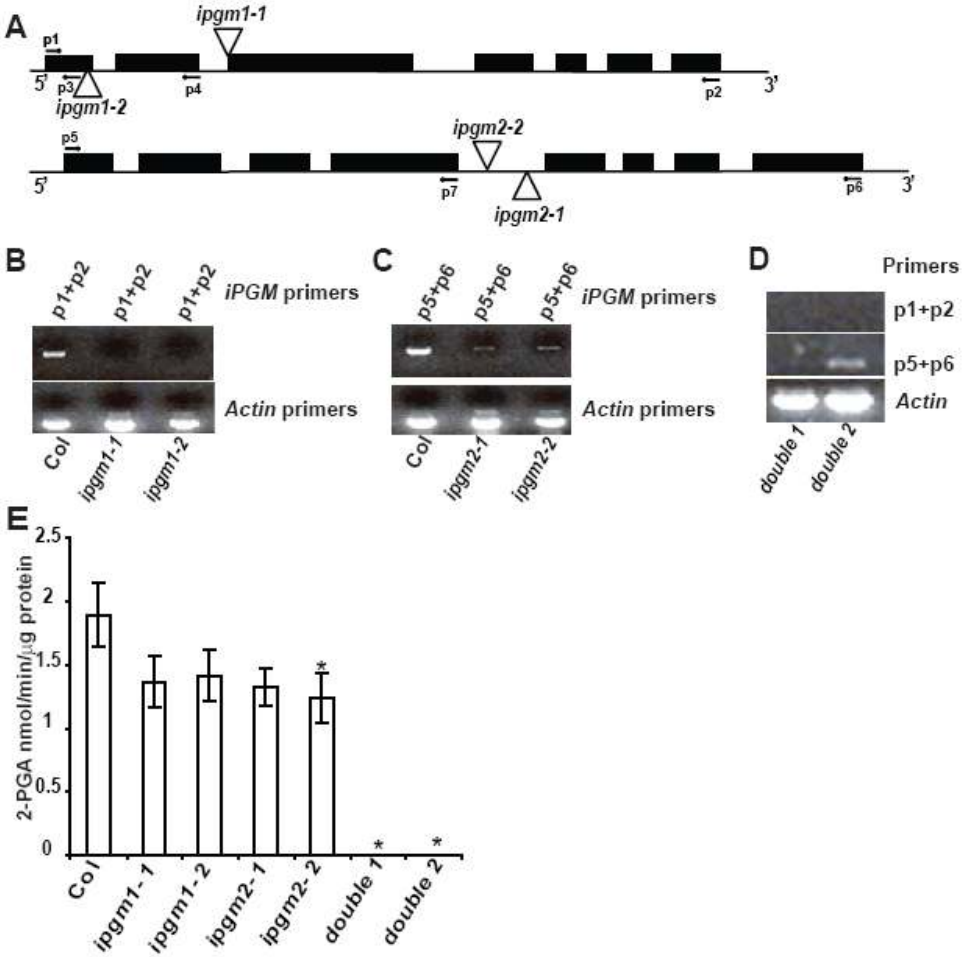



**Figure 2-6**. **Double *ipgm1 ipgm2* mutants have no detectable iPGM enzyme activity.**

(A) *ipgm1-1* or *ipgm1-2* has T-DNA insertion in 3rd or 1st exon of *iPGM1* respectively; *ipgm2-1* and *ipgm2-2* have T-DNA insertions in 4th intron of *iPGM2*.
(B) *ipgm1* mutants are knock-out mutants as indicated by RT-PCR assays using forward primer p1 and reverse primer p2..
(C) *ipgm2* mutants are knock-down mutants as indicated by RT-PCR assays using forward primer p5 and reverse primer p6.
(D) Full length transcript of *iPGM1* is absent in *double 1* and *double 2* mutants (*double 1* = *ipgm1-1 ipgm2-2*, *double 2* = *ipgm1-2 ipgm2-1*), while full-length transcript of *iPGM2* is detected at low levels in *double 2* mutant.
(E) Double mutants have no detectable iPGM enzyme activity. Single mutants have somewhat lower iPGM enzyme activity than Col. n = 4, data are mean ± SE. $P < 0.05$ (Student's t-test) was considered significant (*).

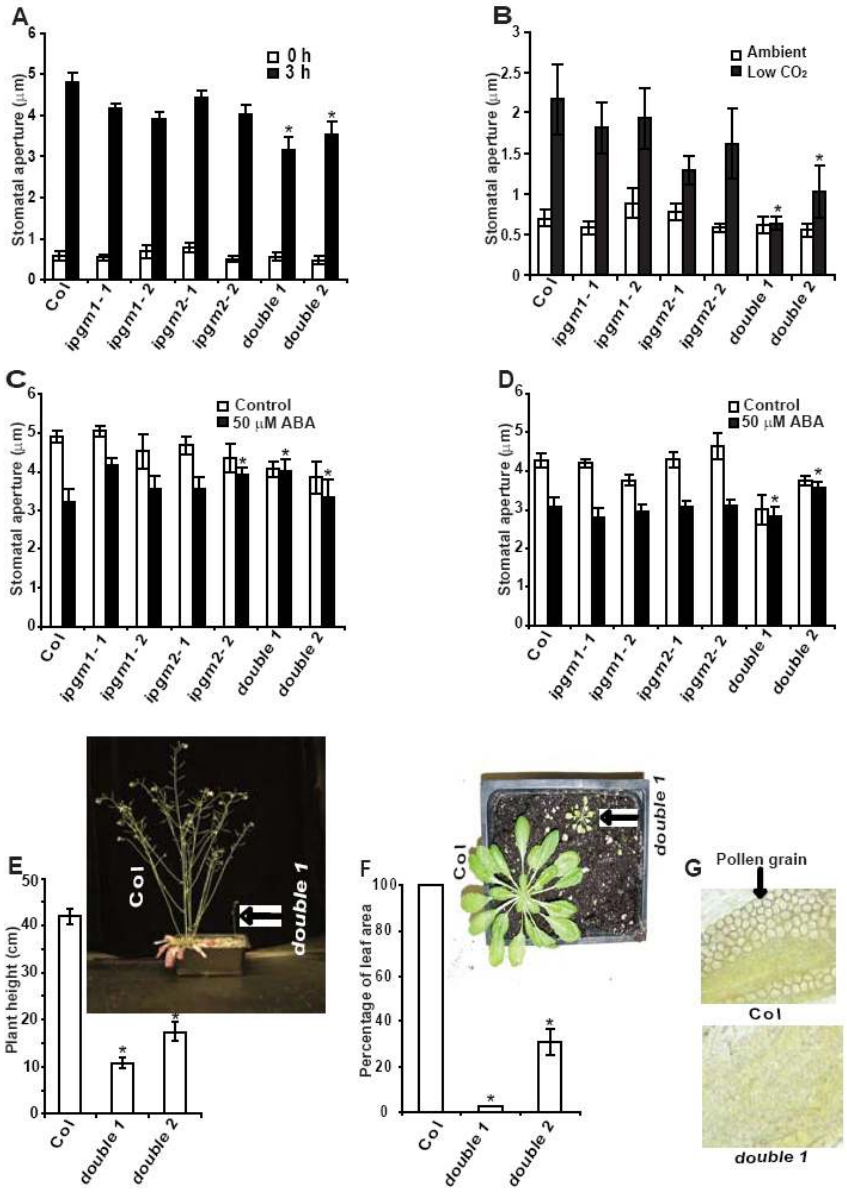



**Figure 2-7. *ipgm1 ipgm2* double mutants have stomatal, vegetative, and reproductive phenotypes**.

(A) Double mutants are hyposensitive to blue light (10 μmol $m^{-2}s^{-1}$) promotion of stomatal opening.
(B) Double mutants are hyposensitive to low $CO_2$ promotion of stomatal opening under darkness.
(C) Double mutants are hyposensitive to ABA (50 μM) promotion of stomatal closure.
(D) Double mutants are hyposensitive to ABA (50 μM) inhibition of stomatal opening.
For (A-D), n= 5, 8, 4 and 4. $P < 0.05$ (Student's t-test) was considered significant (*).
(E) Double mutant plants are dramatically shorter than Col plants.
(F) Double mutant rosette leaf areas are dramatically reduced compared to Col plants.
For (E-F), n = 10. Data are mean ± SE.
(G) No visible pollen grains were found in double mutant anthers. Col and *double 1* plants are shown. The same phenotype was present in *double 2* (data not shown).

**Table 2-1: Proteins identified in our guard cell proteome**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **a. 58 proteins were identified from the BR method.** | | | | | | | |
| At1g06680 | At1g32060 | At1g76030 | At2g39730 | At3g50820 | At4g24770 | At5g27640 | AtCg00490 |
| At1g07570 | At1g51980 | At1g78900 | At2g43750 | At3g55440 | At4g39260 | At5g35630 | AtMg01190 |
| At1g07890 | At1g53240 | At2g20420 | At2g43750 | At3g55800 | At5g08690 | At5g38420 | |
| At1g09210 | At1g55490 | At2g21660 | At3g01500 | At3g61860 | At5g15090 | At5g44340 | |
| At1g09780 | At1g56340 | At2g24200 | At3g04120 | At3g62030 | At5g17310 | At5g44340 | |
| At1g20340 | At1g63940 | At2g28000 | At3g08590 | At4g01850 | At5g17920 | At5g66570 | |
| At1g21750 | At1g67090 | At2g36530 | At3g11630 | At4g02930 | At5g20720 | AtCg00120 | |
| At1g29930 | At1g75280 | At2g37220 | At3g16640 | At4g14960 | At5g23120 | AtCg00480 | |
| **b. 59 proteins were identified from the NR method.** | | | | | | | |
| At1g06680 | At1g32120 | At2g05990 | At2g39730 | At3g50820 | At4g38950 | At5g20720 | At5g66030 |
| At1g13440 | At1g51490 | At2g21170 | At2g43090 | At3g52960 | At4g38970 | At5g26000 | At5g66570 |
| At1g16140 | At1g51980 | At2g21330 | At2g43100 | At3g53460 | At4g39260 | At5g38430 | AtCG00490 |
| At1g17100 | At1g53240 | At2g21660 | At2g46320 | At3g55800 | At4g39730 | At5g44120 | |
| At1g20010 | At1g54040 | At2g28190 | At3g01500 | At3g58990 | At5g08670 | At5g48480 | |
| At1g21750 | At1g56340 | At2g32240 | At3g04120 | At3g61130 | At5g08680 | At5g49910 | |
| At1g29920 | At1g67090 | At2g35490 | At3g15020 | At4g24280 | At5g08690 | At5g57290 | |
| At1g29930 | At1g78370 | At2g37220 | At3g48190 | At4g28520 | At5g15090 | At5g60720 | |
| **c. 750 proteins were identified from the 1st MudPIT using Mascot algorithm.** | | | | | | | |
| At1g01500 | At1g49430 | At2g01350 | At2g44050 | At3g23940 | At4g13770 | At4g39690 | At5g40450 |
| At1g02080 | At1g49630 | At2g01720 | At2g44060 | At3g24160 | At4g13780 | At4g39860 | At5g40490 |
| At1g02840 | At1g49670 | At2g02160 | At2g44200 | At3g24170 | At4g13930 | At5g01530 | At5g41790 |
| At1g03090 | At1g49820 | At2g05070 | At2g44490 | At3g24420 | At4g13940 | At5g02960 | At5g41970 |
| At1g03190 | At1g50030 | At2g05710 | At2g44525 | At3g24480 | At4g14040 | At5g03340 | At5g42390 |
| At1g03475 | At1g50200 | At2g05830 | At2g44640 | At3g26060 | At4g14360 | At5g03630 | At5g42820 |
| At1g04170 | At1g50380 | At2g05840 | At2g45300 | At3g26340 | At4g14440 | At5g03740 | At5g42950 |
| At1g04410 | At1g51710 | At2g05990 | At2g45540 | At3g26400 | At4g14480 | At5g04140 | At5g43130 |
| At1g04430 | At1g52400 | At2g06210 | At2g45600 | At3g26650 | At4g14680 | At5g04430 | At5g43330 |
| At1g04510 | At1g52730 | At2g07050 | At2g45810 | At3g27925 | At4g14880 | At5g04590 | At5g44100 |
| At1g06630 | At1g53000 | At2g13360 | At2g46020 | At3g28530 | At4g14960 | At5g04710 | At5g45160 |
| At1g06680 | At1g53210 | At2g13560 | At2g46070 | At3g28730 | At4g15020 | At5g05170 | At5g46290 |
| At1g07360 | At1g53290 | At2g14720 | At2g46280 | At3g42050 | At4g16130 | At5g06110 | At5g46570 |
| At1g07370 | At1g53310 | At2g15620 | At2g46290 | At3g43300 | At4g16190 | At5g06390 | At5g47040 |
| At1g07780 | At1g53580 | At2g16600 | At2g47390 | At3g44310 | At4g17330 | At5g06530 | At5g47650 |
| At1g07810 | At1g54040 | At2g16890 | At2g47510 | At3g44330 | At4g17830 | At5g07300 | At5g47860 |
| At1g07890 | At1g54170 | At2g17980 | At3g01120 | At3g45980 | At4g18030 | At5g07350 | At5g47870 |
| At1g07920 | At1g54560 | At2g18450 | At3g01180 | At3g46740 | At4g18820 | At5g08280 | At5g47930 |
| At1g08420 | At1g54610 | At2g18960 | At3g01280 | At3g46970 | At4g19120 | At5g08300 | At5g48375 |
| At1g08470 | At1g55860 | At2g19080 | At3g01310 | At3g47520 | At4g19210 | At5g08450 | At5g48440 |
| At1g08520 | At1g56070 | At2g19520 | At3g01480 | At3g47590 | At4g20360 | At5g08540 | At5g48480 |
| At1g08980 | At1g56450 | At2g20420 | At3g01500 | At3g47650 | At4g20850 | At5g08680 | At5g49460 |
| At1g09210 | At1g57620 | At2g20990 | At3g01670 | At3g47810 | At4g20890 | At5g09810 | At5g49555 |
| At1g09340 | At1g58380 | At2g21060 | At3g01680 | At3g48000 | At4g20960 | At5g10010 | At5g49910 |
| At1g09780 | At1g59870 | At2g21130 | At3g02080 | At3g48410 | At4g21150 | At5g10360 | At5g50230 |

| At1g09795 | At1g61580 | At2g21160 | At3g02090 | At3g48430 | At4g21160 | At5g10540 | At5g50920 |
|---|---|---|---|---|---|---|---|
| At1g10430 | At1g61770 | At2g21410 | At3g02350 | At3g48720 | At4g22150 | At5g10730 | At5g50950 |
| At1g10770 | At1g62020 | At2g21530 | At3g03780 | At3g48730 | At4g22690 | At5g10860 | At5g51200 |
| At1g10950 | At1g62330 | At2g21590 | At3g03960 | At3g48870 | At4g22720 | At5g11200 | At5g52320 |
| At1g11260 | At1g62360 | At2g21660 | At3g04120 | At3g48990 | At4g23630 | At5g11560 | At5g52920 |
| At1g11860 | At1g62750 | At2g21880 | At3g04610 | At3g49010 | At4g23650 | At5g11670 | At5g53460 |
| At1g12470 | At1g63000 | At2g22125 | At3g04840 | At3g49120 | At4g23730 | At5g11880 | At5g53480 |
| At1g13020 | At1g63180 | At2g22400 | At3g05060 | At3g49680 | At4g24510 | At5g12250 | At5g53850 |
| At1g13060 | At1g63660 | At2g22480 | At3g05280 | At3g50370 | At4g24620 | At5g12960 | At5g54810 |
| At1g13930 | At1g63770 | At2g23350 | At3g06510 | At3g50590 | At4g24800 | At5g13480 | At5g54900 |
| At1g14010 | At1g63940 | At2g23600 | At3g06580 | At3g52200 | At4g24830 | At5g13520 | At5g54960 |
| At1g14830 | At1g63970 | At2g24180 | At3g06800 | At3g52850 | At4g25130 | At5g13640 | At5g55040 |
| At1g14850 | At1g64190 | At2g24200 | At3g06850 | At3g52880 | At4g25960 | At5g13710 | At5g55670 |
| At1g15340 | At1g64550 | At2g24270 | At3g06860 | At3g52930 | At4g26630 | At5g13850 | At5g57110 |
| At1g15730 | At1g64760 | At2g24420 | At3g06980 | At3g52990 | At4g26900 | At5g13980 | At5g57460 |
| At1g15750 | At1g65440 | At2g24580 | At3g07140 | At3g53580 | At4g26910 | At5g14740 | At5g58270 |
| At1g17840 | At1g65930 | At2g24590 | At3g07220 | At3g54050 | At4g26970 | At5g14780 | At5g58410 |
| At1g18080 | At1g66200 | At2g25110 | At3g08510 | At3g54300 | At4g27000 | At5g15090 | At5g58870 |
| At1g18210 | At1g67090 | At2g25170 | At3g08530 | At3g54640 | At4g27430 | At5g15200 | At5g59370 |
| At1g18270 | At1g67140 | At2g25450 | At3g08590 | At3g54760 | At4g27560 | At5g15350 | At5g59750 |
| At1g18450 | At1g67490 | At2g25560 | At3g08680 | At3g55140 | At4g29010 | At5g15400 | At5g59820 |
| At1g18590 | At1g67580 | At2g26040 | At3g08740 | At3g55200 | At4g29060 | At5g16510 | At5g59910 |
| At1g19130 | At1g67640 | At2g26140 | At3g09440 | At3g55410 | At4g29120 | At5g16590 | At5g60540 |
| At1g19570 | At1g68010 | At2g26680 | At3g09630 | At3g55440 | At4g29810 | At5g16780 | At5g60640 |
| At1g20010 | At1g68530 | At2g26730 | At3g09820 | At3g55800 | At4g29900 | At5g17050 | At5g60980 |
| At1g20020 | At1g68830 | At2g26890 | At3g10380 | At3g55830 | At4g30530 | At5g17380 | At5g61150 |
| At1g20340 | At1g69340 | At2g27040 | At3g10670 | At3g57030 | At4g30800 | At5g17770 | At5g61640 |
| At1g20620 | At1g69830 | At2g27100 | At3g10690 | At3g57300 | At4g31130 | At5g17920 | At5g61780 |
| At1g20950 | At1g70410 | At2g28450 | At3g10920 | At3g57890 | At4g31340 | At5g18100 | At5g61790 |
| At1g20960 | At1g70490 | At2g30050 | At3g11130 | At3g58140 | At4g31490 | At5g19220 | At5g62020 |
| At1g20970 | At1g70600 | At2g30200 | At3g11170 | At3g58510 | At4g31580 | At5g19290 | At5g62530 |
| At1g21160 | At1g70770 | At2g30720 | At3g11210 | At3g59020 | At4g31700 | At5g19370 | At5g62670 |
| At1g21540 | At1g71410 | At2g30950 | At3g11400 | At3g60600 | At4g31770 | At5g19440 | At5g63260 |
| At1g21680 | At1g71840 | At2g30970 | At3g11630 | At3g60750 | At4g32280 | At5g19510 | At5g63550 |
| At1g21750 | At1g71860 | At2g32240 | At3g12810 | At3g60770 | At4g32410 | At5g19620 | At5g63570 |
| At1g22410 | At1g72150 | At2g32410 | At3g13300 | At3g60830 | At4g32640 | At5g19690 | At5g63620 |
| At1g22920 | At1g72160 | At2g32520 | At3g13460 | At3g61240 | At4g32770 | At5g19770 | At5g63860 |
| At1g23190 | At1g72370 | At2g32920 | At3g13870 | At3g61690 | At4g33010 | At5g19820 | At5g64350 |
| At1g23290 | At1g72610 | At2g33040 | At3g14067 | At3g62030 | At4g33030 | At5g19990 | At5g64370 |
| At1g24706 | At1g72680 | At2g33150 | At3g14120 | At3g63260 | At4g33090 | At5g20020 | At5g64740 |
| At1g26550 | At1g73370 | At2g33730 | At3g14420 | At3g63410 | At4g33150 | At5g20290 | At5g65750 |
| At1g26850 | At1g73670 | At2g34660 | At3g14570 | At3g63460 | At4g33650 | At5g20720 | At5g65760 |
| At1g27090 | At1g74260 | At2g35010 | At3g15020 | At3g63520 | At4g33680 | At5g22640 | At5g66040 |
| At1g27430 | At1g74470 | At2g35040 | At3g15090 | At4g00570 | At4g34180 | At5g22650 | At5g66420 |
| At1g27680 | At1g74910 | At2g35050 | At3g15730 | At4g00585 | At4g34240 | At5g22740 | At5g66530 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| At1g28060 | At1g74920 | At2g35100 | At3g15980 | At4g01050 | At4g34490 | At5g23120 | At5g66570 |
| At1g28420 | At1g75640 | At2g35530 | At3g16110 | At4g01310 | At4g34740 | At5g23900 | At5g66680 |
| At1g29910 | At1g75660 | At2g35790 | At3g16170 | At4g01370 | At4g35300 | At5g24300 | At5g67500 |
| At1g30000 | At1g75680 | At2g35850 | At3g16270 | At4g01850 | At4g35800 | At5g26000 | At5g67610 |
| At1g30360 | At1g76400 | At2g36060 | At3g16850 | At4g02080 | At4g35830 | At5g26710 | AtCg00020 |
| At1g30400 | At1g76550 | At2g36530 | At3g17240 | At4g02930 | At4g36130 | At5g26742 | AtCg00120 |
| At1g30580 | At1g76690 | At2g36880 | At3g17390 | At4g03550 | At4g36250 | At5g26860 | AtCg00170 |
| At1g31190 | At1g76720 | At2g38270 | At3g17810 | At4g04320 | At4g37000 | At5g27630 | AtCG00270 |
| At1g31330 | At1g76850 | At2g38280 | At3g17910 | At4g04910 | At4g37300 | At5g28540 | AtCg00280 |
| At1g31850 | At1g77260 | At2g38770 | At3g17940 | At4g05530 | At4g37550 | At5g28840 | AtCg00340 |
| At1g32060 | At1g77510 | At2g39730 | At3g18040 | At4g05590 | At4g37870 | At5g34850 | AtCg00350 |
| At1g32470 | At1g77940 | At2g39800 | At3g18520 | At4g08810 | At4g37930 | At5g35360 | AtCg00480 |
| At1g33140 | At1g78580 | At2g39850 | At3g18780 | At4g09980 | At4g38350 | At5g35430 | AtCg00490 |
| At1g34220 | At1g78630 | At2g40490 | At3g19760 | At4g10320 | At4g38460 | At5g35630 | AtCg00680 |
| At1g35160 | At1g78880 | At2g40840 | At3g19870 | At4g10340 | At4g38510 | At5g37510 | AtCg00720 |
| At1g42550 | At1g78900 | At2g41220 | At3g20000 | At4g11010 | At4g38680 | At5g37600 | AtCg00800 |
| At1g42970 | At1g79230 | At2g41560 | At3g20300 | At4g11380 | At4g38740 | At5g38420 | AtCg00830 |
| At1g43170 | At1g79340 | At2g41630 | At3g21110 | At4g11420 | At4g38800 | At5g38470 | AtCg00860 |
| At1g43670 | At1g79530 | At2g42520 | At3g21865 | At4g11560 | At4g38900 | At5g38530 | AtMg00090 |
| At1g44575 | At1g79550 | At2g42600 | At3g22110 | At4g12420 | At4g38970 | At5g39080 | AtMg00480 |
| At1g45201 | At1g79990 | At2g43160 | At3g22460 | At4g12610 | At4g39080 | At5g39410 | AtMg00510 |
| At1g48030 | At1g80070 | At2g43770 | At3g22960 | At4g12780 | At4g39280 | At5g39500 | AtMg01190 |
| At1g48620 | At1g80410 | At2g43910 | At3g23410 | At4g12800 | At4g39330 | At5g39570 | |
| At1g49040 | At1g80480 | At2g43940 | At3g23820 | At4g13340 | At4g39660 | At5g40340 | |
| **d. 1192 proteins were identified from the 1st MudPIT using the Protein Pilot algorithm.** | | | | | | | |
| At1g01510 | At1g45201 | At2g04880 | At2g44100 | At3g26720 | At4g12610 | At4g38900 | At5g39500 |
| At1g01960 | At1g47128 | At2g05100 | At2g44160 | At3g27230 | At4g12770 | At4g38970 | At5g39570 |
| At1g02080 | At1g47260 | At2g05710 | At2g44200 | At3g27300 | At4g12800 | At4g39080 | At5g40340 |
| At1g02500 | At1g47710 | At2g05830 | At2g44490 | At3g27925 | At4g13430 | At4g39090 | At5g40450 |
| At1g02840 | At1g48030 | At2g05840 | At2g44525 | At3g28720 | At4g13770 | At4g39260 | At5g40490 |
| At1g02890 | At1g48270 | At2g05990 | At2g44640 | At3g28730 | At4g13780 | At4g39280 | At5g40650 |
| At1g03090 | At1g48410 | At2g06050 | At2g44920 | At3g29075 | At4g13850 | At4g39330 | At5g40780 |
| At1g03190 | At1g48600 | At2g06210 | At2g45140 | At3g29320 | At4g13930 | At4g39660 | At5g40890 |
| At1g03475 | At1g48605 | At2g07050 | At2g45300 | At3g42050 | At4g13940 | At4g39680 | At5g41670 |
| At1g03910 | At1g48620 | At2g07360 | At2g45540 | At3g42170 | At4g14040 | At4g39690 | At5g41790 |
| At1g04080 | At1g48850 | At2g12400 | At2g45600 | At3g42950 | At4g14360 | At4g39730 | At5g41970 |
| At1g04170 | At1g48920 | At2g13360 | At2g45790 | At3g43300 | At4g14440 | At4g39860 | At5g42020 |
| At1g04270 | At1g49240 | At2g13370 | At2g45810 | At3g44110 | At4g14480 | At4g39960 | At5g42080 |
| At1g04410 | At1g49430 | At2g13560 | At2g45880 | At3g44190 | At4g14570 | At5g01460 | At5g42270 |
| At1g04430 | At1g49670 | At2g14170 | At2g46020 | At3g44310 | At4g14880 | At5g01530 | At5g42390 |
| At1g04600 | At1g49760 | At2g14720 | At2g46070 | At3g44330 | At4g14960 | At5g01920 | At5g42820 |
| At1g05500 | At1g50010 | At2g15620 | At2g46280 | At3g44600 | At4g15020 | At5g02100 | At5g42950 |
| At1g05910 | At1g50030 | At2g16600 | At2g47240 | At3g45140 | At4g15900 | At5g02490 | At5g42960 |
| At1g06030 | At1g50200 | At2g16890 | At2g47390 | At3g45190 | At4g16130 | At5g02500 | At5g43130 |
| At1g06130 | At1g50380 | At2g17130 | At2g47470 | At3g45780 | At4g16143 | At5g02960 | At5g43320 |

| At1g06190 | At1g50480 | At2g17390 | At2g47510 | At3g45980 | At4g16190 | At5g03630 | At5g43330 |
|---|---|---|---|---|---|---|---|
| At1g06430 | At1g51710 | At2g17410 | At2g47710 | At3g46040 | At4g16590 | At5g03740 | At5g43760 |
| At1g06550 | At1g51980 | At2g17930 | At2g47980 | At3g46740 | At4g16760 | At5g04060 | At5g43830 |
| At1g06570 | At1g52360 | At2g17980 | At3g01120 | At3g46960 | At4g17100 | At5g04110 | At5g43940 |
| At1g06630 | At1g52380 | At2g18020 | At3g01180 | At3g46970 | At4g17140 | At5g04140 | At5g44320 |
| At1g06680 | At1g52400 | At2g18960 | At3g01280 | At3g47470 | At4g17330 | At5g04430 | At5g45160 |
| At1g06690 | At1g52600 | At2g19520 | At3g01310 | At3g47520 | At4g17390 | At5g04440 | At5g45930 |
| At1g06950 | At1g52730 | At2g19730 | At3g01340 | At3g47590 | At4g17600 | At5g04590 | At5g46070 |
| At1g07040 | At1g53000 | At2g19760 | At3g01480 | At3g47650 | At4g17620 | At5g04710 | At5g46110 |
| At1g07360 | At1g53210 | At2g19860 | At3g01500 | At3g47810 | At4g17770 | At5g05010 | At5g46290 |
| At1g07670 | At1g53240 | At2g19940 | At3g01670 | At3g47930 | At4g17830 | At5g05170 | At5g46570 |
| At1g07780 | At1g53290 | At2g20190 | At3g01680 | At3g48000 | At4g18030 | At5g05670 | At5g46750 |
| At1g07790 | At1g53310 | At2g20260 | At3g01780 | At3g48410 | At4g18670 | At5g05680 | At5g47040 |
| At1g07890 | At1g53580 | At2g20420 | At3g01980 | At3g48425 | At4g18810 | At5g05740 | At5g47190 |
| At1g07930 | At1g53720 | At2g20610 | At3g02090 | At3g48430 | At4g18820 | At5g06110 | At5g47640 |
| At1g08200 | At1g54040 | At2g20990 | At3g02180 | At3g48560 | At4g19120 | At5g06390 | At5g47650 |
| At1g08470 | At1g54170 | At2g21060 | At3g02350 | At3g48720 | At4g19210 | At5g06600 | At5g47690 |
| At1g08520 | At1g54570 | At2g21130 | At3g02360 | At3g48730 | At4g19710 | At5g07090 | At5g47860 |
| At1g08830 | At1g54610 | At2g21160 | At3g03710 | At3g48870 | At4g20360 | At5g07300 | At5g47870 |
| At1g08980 | At1g54990 | At2g21170 | At3g03780 | At3g48990 | At4g20400 | At5g07350 | At5g48180 |
| At1g09070 | At1g55320 | At2g21390 | At3g04120 | At3g49010 | At4g20760 | At5g07440 | At5g48300 |
| At1g09210 | At1g55670 | At2g21410 | At3g04470 | At3g49430 | At4g20850 | At5g08280 | At5g48375 |
| At1g09270 | At1g55860 | At2g21530 | At3g04610 | At3g49600 | At4g20960 | At5g08300 | At5g48440 |
| At1g09340 | At1g55900 | At2g21590 | At3g04740 | At3g49680 | At4g21100 | At5g08450 | At5g48480 |
| At1g09590 | At1g56070 | At2g21660 | At3g04770 | At3g49720 | At4g21150 | At5g08530 | At5g49460 |
| At1g09780 | At1g56110 | At2g21880 | At3g04790 | At3g50370 | At4g21160 | At5g08550 | At5g49555 |
| At1g09795 | At1g56340 | At2g22125 | At3g04840 | At3g50590 | At4g21180 | At5g08580 | At5g49760 |
| At1g10200 | At1g57620 | At2g22360 | At3g04870 | At3g50670 | At4g21280 | At5g08680 | At5g49910 |
| At1g10390 | At1g58025 | At2g22400 | At3g04920 | At3g51610 | At4g21710 | At5g09590 | At5g50230 |
| At1g10670 | At1g59610 | At2g22480 | At3g05030 | At3g52200 | At4g21960 | At5g09810 | At5g50920 |
| At1g10770 | At1g59820 | At2g22900 | At3g05060 | At3g52380 | At4g22150 | At5g10010 | At5g50950 |
| At1g10950 | At1g59870 | At2g23600 | At3g05420 | At3g52560 | At4g22690 | At5g10360 | At5g51200 |
| At1g11260 | At1g59900 | At2g24180 | At3g06410 | At3g52580 | At4g22720 | At5g10470 | At5g51820 |
| At1g11840 | At1g61770 | At2g24200 | At3g06510 | At3g52590 | At4g23460 | At5g10730 | At5g52310 |
| At1g11860 | At1g61790 | At2g24270 | At3g06580 | At3g52850 | At4g23630 | At5g10860 | At5g52320 |
| At1g11890 | At1g62330 | At2g24420 | At3g06650 | At3g52880 | At4g23650 | At5g11060 | At5g52470 |
| At1g12000 | At1g62390 | At2g24520 | At3g06810 | At3g52930 | At4g23730 | At5g11170 | At5g53480 |
| At1g12240 | At1g62560 | At2g24580 | At3g06850 | At3g52960 | At4g23850 | At5g11560 | At5g53850 |
| At1g12470 | At1g62740 | At2g24590 | At3g06860 | At3g52990 | At4g23900 | At5g11670 | At5g54440 |
| At1g13020 | At1g62750 | At2g25110 | At3g06980 | At3g53020 | At4g23920 | At5g11700 | At5g54810 |
| At1g13060 | At1g63010 | At2g25170 | At3g07100 | At3g53130 | At4g24190 | At5g11710 | At5g54900 |
| At1g13090 | At1g63180 | At2g25450 | At3g07140 | At3g53460 | At4g24220 | At5g11770 | At5g54960 |
| At1g14010 | At1g63310 | At2g25560 | At3g07220 | At3g53520 | At4g24280 | At5g12040 | At5g55040 |
| At1g14820 | At1g63660 | At2g25670 | At3g07660 | At3g53580 | At4g24510 | At5g12130 | At5g55220 |
| At1g14830 | At1g63770 | At2g26040 | At3g08510 | At3g53720 | At4g24620 | At5g12250 | At5g55660 |

| At1g14850 | At1g63940 | At2g26080 | At3g08530 | At3g54050 | At4g24680 | At5g12960 | At5g55670 |
|---|---|---|---|---|---|---|---|
| At1g15000 | At1g63970 | At2g26140 | At3g08580 | At3g54300 | At4g24770 | At5g13010 | At5g55960 |
| At1g15340 | At1g64090 | At2g26680 | At3g08590 | At3g54440 | At4g24800 | At5g13420 | At5g56030 |
| At1g15750 | At1g64550 | At2g26730 | At3g08680 | At3g54640 | At4g24830 | At5g13440 | At5g56360 |
| At1g15820 | At1g64760 | At2g26890 | At3g08740 | At3g54760 | At4g25130 | At5g13520 | At5g56500 |
| At1g17160 | At1g65440 | At2g26900 | At3g08940 | At3g55010 | At4g25960 | At5g13640 | At5g57110 |
| At1g17290 | At1g65930 | At2g26910 | At3g09440 | At3g55020 | At4g26300 | At5g13710 | At5g57330 |
| At1g17470 | At1g66200 | At2g27040 | At3g09630 | At3g55140 | At4g26630 | At5g13850 | At5g57350 |
| At1g17840 | At1g67090 | At2g27100 | At3g09820 | At3g55200 | At4g26720 | At5g13930 | At5g57460 |
| At1g18070 | At1g67140 | At2g27190 | At3g09840 | At3g55260 | At4g26900 | At5g13980 | At5g57490 |
| At1g18210 | At1g67230 | At2g27210 | At3g10380 | At3g55360 | At4g26910 | At5g14040 | At5g57870 |
| At1g18270 | At1g67280 | At2g27450 | At3g10670 | At3g55410 | At4g26970 | At5g14120 | At5g58040 |
| At1g18440 | At1g67490 | At2g28000 | At3g10690 | At3g55440 | At4g27000 | At5g14740 | At5g58100 |
| At1g18500 | At1g67580 | At2g28190 | At3g10740 | At3g55750 | At4g27230 | At5g14780 | At5g58140 |
| At1g18590 | At1g68010 | At2g28290 | At3g10920 | At3g55800 | At4g27430 | At5g15090 | At5g58270 |
| At1g19130 | At1g68530 | At2g28450 | At3g11130 | At3g55830 | At4g27440 | At5g15200 | At5g58330 |
| At1g19370 | At1g68830 | At2g29550 | At3g11170 | At3g56150 | At4g27560 | At5g15210 | At5g58410 |
| At1g19580 | At1g69250 | At2g29980 | At3g11210 | At3g56750 | At4g27640 | At5g15350 | At5g58870 |
| At1g19870 | At1g69340 | At2g30050 | At3g11400 | At3g56820 | At4g28080 | At5g15400 | At5g59370 |
| At1g20010 | At1g69460 | At2g30110 | At3g11450 | At3g57030 | At4g28750 | At5g15450 | At5g59420 |
| At1g20020 | At1g69830 | At2g30200 | At3g11630 | At3g57300 | At4g29010 | At5g15520 | At5g59750 |
| At1g20200 | At1g70410 | At2g30490 | At3g11910 | At3g57610 | At4g29060 | At5g15680 | At5g59820 |
| At1g20260 | At1g70580 | At2g30620 | At3g12580 | At3g57890 | At4g29480 | At5g16510 | At5g59910 |
| At1g20340 | At1g70600 | At2g30720 | At3g12810 | At3g57990 | At4g29680 | At5g16590 | At5g59950 |
| At1g20620 | At1g70770 | At2g30950 | At3g13300 | At3g58140 | At4g29810 | At5g16780 | At5g60160 |
| At1g20840 | At1g71220 | At2g30970 | At3g13330 | At3g58510 | At4g29840 | At5g17050 | At5g60360 |
| At1g20950 | At1g71310 | At2g31660 | At3g13450 | At3g58610 | At4g29900 | At5g17170 | At5g60390 |
| At1g20960 | At1g71410 | At2g32240 | At3g13460 | At3g58750 | At4g30530 | At5g17380 | At5g60540 |
| At1g20970 | At1g72160 | At2g32410 | At3g13570 | At3g59500 | At4g30720 | At5g17770 | At5g60980 |
| At1g21160 | At1g72500 | At2g32520 | At3g13870 | At3g59780 | At4g30800 | At5g17920 | At5g61450 |
| At1g21540 | At1g72610 | At2g32730 | At3g14067 | At3g59920 | At4g31130 | At5g18100 | At5g61640 |
| At1g21680 | At1g72680 | At2g32910 | At3g14100 | At3g59970 | At4g31160 | At5g18485 | At5g61780 |
| At1g21750 | At1g73370 | At2g32920 | At3g14120 | At3g59990 | At4g31180 | At5g19220 | At5g61790 |
| At1g22410 | At1g73670 | At2g33150 | At3g14390 | At3g60240 | At4g31340 | At5g19290 | At5g62390 |
| At1g22430 | At1g74260 | At2g33340 | At3g14415 | At3g60600 | At4g31420 | At5g19370 | At5g62530 |
| At1g22920 | At1g74470 | At2g33730 | At3g14420 | At3g60750 | At4g31480 | At5g19440 | At5g62670 |
| At1g23190 | At1g74910 | At2g34430 | At3g15090 | At3g60830 | At4g31500 | At5g19510 | At5g62690 |
| At1g23820 | At1g74920 | At2g34660 | At3g15730 | At3g61240 | At4g31580 | At5g19550 | At5g62790 |
| At1g24160 | At1g74960 | At2g35010 | At3g15980 | At3g61440 | At4g31700 | At5g19620 | At5g63040 |
| At1g24180 | At1g75040 | At2g35040 | At3g16080 | At3g61650 | At4g31770 | At5g19690 | At5g63260 |
| At1g24706 | At1g75130 | At2g35050 | At3g16110 | At3g61690 | At4g31880 | At5g19760 | At5g63510 |
| At1g25350 | At1g75330 | At2g35110 | At3g16170 | At3g62030 | At4g31990 | At5g19770 | At5g63550 |
| At1g26110 | At1g75640 | At2g35530 | At3g16640 | At3g62110 | At4g32250 | At5g19820 | At5g63570 |
| At1g26550 | At1g75660 | At2g35790 | At3g16850 | At3g63140 | At4g32285 | At5g19990 | At5g63620 |
| At1g26630 | At1g75680 | At2g35810 | At3g16910 | At3g63260 | At4g32410 | At5g20020 | At5g63840 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| At1g26850 | At1g75780 | At2g35840 | At3g17240 | At3g63410 | At4g32640 | At5g20080 | At5g63860 |
| At1g26910 | At1g76080 | At2g36290 | At3g17390 | At3g63460 | At4g32770 | At5g20280 | At5g64270 |
| At1g26940 | At1g76180 | At2g36390 | At3g17810 | At3g63490 | At4g32940 | At5g20290 | At5g64350 |
| At1g27090 | At1g76400 | At2g36460 | At3g17910 | At3g63520 | At4g33010 | At5g20720 | At5g64370 |
| At1g27430 | At1g76680 | At2g36530 | At3g17940 | At3g66654 | At4g33030 | At5g20980 | At5g64400 |
| At1g27680 | At1g76850 | At2g36620 | At3g18040 | At4g00100 | At4g33090 | At5g21160 | At5g64740 |
| At1g28420 | At1g76880 | At2g36850 | At3g18520 | At4g00360 | At4g33150 | At5g22640 | At5g65620 |
| At1g29370 | At1g77260 | At2g36880 | At3g18990 | At4g00430 | At4g33580 | At5g22650 | At5g65750 |
| At1g29400 | At1g77510 | At2g37220 | At3g19170 | At4g00570 | At4g33650 | At5g23120 | At5g65760 |
| At1g29470 | At1g77940 | At2g37660 | At3g19710 | At4g00640 | At4g34110 | At5g23860 | At5g65770 |
| At1g29690 | At1g78570 | At2g38270 | At3g19760 | At4g01050 | At4g34200 | At5g23900 | At5g65930 |
| At1g29880 | At1g78580 | At2g38280 | At3g19820 | At4g01310 | At4g34240 | At5g24300 | At5g66040 |
| At1g29900 | At1g78630 | At2g38290 | At3g19870 | At4g01370 | At4g34430 | At5g24710 | At5g66190 |
| At1g30120 | At1g78880 | At2g38670 | At3g19930 | At4g01810 | At4g34450 | At5g25150 | At5g66400 |
| At1g30230 | At1g78900 | At2g38730 | At3g19980 | At4g02080 | At4g34490 | At5g26000 | At5g66420 |
| At1g30360 | At1g79040 | At2g38770 | At3g20300 | At4g02280 | At4g34740 | At5g26280 | At5g66530 |
| At1g30470 | At1g79210 | At2g39730 | At3g20560 | At4g02420 | At4g34950 | At5g26710 | At5g66570 |
| At1g30510 | At1g79230 | At2g39800 | At3g21110 | At4g02510 | At4g35300 | At5g26742 | At5g66680 |
| At1g30580 | At1g79280 | At2g39850 | At3g21865 | At4g02580 | At4g35790 | At5g26830 | At5g66760 |
| At1g31070 | At1g79440 | At2g39990 | At3g22110 | At4g02930 | At4g35800 | At5g26860 | At5g67500 |
| At1g31190 | At1g79530 | At2g40490 | At3g22170 | At4g03550 | At4g35830 | At5g27540 | At5g67510 |
| At1g31330 | At1g79550 | At2g40840 | At3g22200 | At4g04340 | At4g36130 | At5g27640 | At5g67610 |
| At1g31710 | At1g79920 | At2g41220 | At3g22230 | At4g04610 | At4g36190 | At5g28840 | AtCg00120 |
| At1g31730 | At1g79990 | At2g41530 | At3g22890 | At4g04910 | At4g36250 | At5g34850 | AtCg00170 |
| At1g31850 | At1g80070 | At2g41560 | At3g22960 | At4g05530 | At4g36690 | At5g35360 | AtCG00270 |
| At1g32060 | At1g80410 | At2g41630 | At3g23410 | At4g05590 | At4g37000 | At5g35430 | AtCG00280 |
| At1g32220 | At1g80930 | At2g41740 | At3g23700 | At4g07830 | At4g37300 | At5g35590 | AtCg00340 |
| At1g32470 | At2g01140 | At2g41790 | At3g23750 | At4g08310 | At4g37550 | At5g35630 | AtCg00350 |
| At1g33140 | At2g01250 | At2g41840 | At3g23810 | At4g08350 | At4g37870 | At5g36210 | AtCg00480 |
| At1g34220 | At2g01350 | At2g42220 | At3g23820 | At4g09000 | At4g37910 | At5g36790 | AtCg00490 |
| At1g34430 | At2g01410 | At2g42500 | At3g23840 | At4g09980 | At4g37930 | At5g36880 | AtCg00540 |
| At1g36160 | At2g01470 | At2g42520 | At3g24160 | At4g10320 | At4g38130 | At5g37510 | AtCg00680 |
| At1g37130 | At2g01690 | At2g42600 | At3g24170 | At4g10340 | At4g38350 | At5g37600 | AtCg00830 |
| At1g42550 | At2g01970 | At2g42710 | At3g24420 | At4g10710 | At4g38460 | At5g37830 | AtCg00860 |
| At1g42970 | At2g02160 | At2g43750 | At3g25230 | At4g11420 | At4g38600 | At5g38410 | AtCg01000 |
| At1g43170 | At2g02540 | At2g43770 | At3g26060 | At4g11560 | At4g38680 | At5g38470 | AtMg00090 |
| At1g43670 | At2g03820 | At2g43940 | At3g26400 | At4g11600 | At4g38690 | At5g38530 | AtMg00480 |
| At1g44575 | At2g04030 | At2g44050 | At3g26430 | At4g11660 | At4g38740 | At5g39040 | AtMg00510 |
| At1g44820 | At2g04280 | At2g44060 | At3g26650 | At4g12420 | At4g38800 | At5g39410 | AtMg01190 |
| **e. 912 proteins were identified from the 2nd MudPIT using the Mascot algorithm.** | | | | | | | |
| At1g01080 | At1g43170 | At2g09990 | At2g44160 | At3g26720 | At4g12800 | At5g02020 | At5g39410 |
| At1g01510 | At1g43670 | At2g13560 | At2g44640 | At3g26740 | At4g13430 | At5g02240 | At5g39570 |
| At1g01610 | At1g44575 | At2g14170 | At2g44920 | At3g26935 | At4g13770 | At5g02490 | At5g40340 |
| At1g02080 | At1g44910 | At2g14720 | At2g45290 | At3g27240 | At4g13840 | At5g02500 | At5g40450 |
| At1g02140 | At1g45201 | At2g15620 | At2g45300 | At3g27300 | At4g13850 | At5g02960 | At5g40490 |

| At1g02280 | At1g47128 | At2g16485 | At2g45440 | At3g28730 | At4g13930 | At5g03040 | At5g40760 |
|---|---|---|---|---|---|---|---|
| At1g02500 | At1g47260 | At2g16600 | At2g45540 | At3g29075 | At4g13940 | At5g03300 | At5g40810 |
| At1g02560 | At1g47420 | At2g16940 | At2g46070 | At3g29320 | At4g14040 | At5g03340 | At5g42020 |
| At1g02780 | At1g48030 | At2g17420 | At2g46280 | At3g29360 | At4g14070 | At5g03560 | At5g42270 |
| At1g03475 | At1g48410 | At2g18020 | At2g47240 | At3g29630 | At4g14360 | At5g03630 | At5g42790 |
| At1g03630 | At1g48600 | At2g18960 | At2g47390 | At3g42170 | At4g14440 | At5g04140 | At5g42820 |
| At1g03680 | At1g48605 | At2g19520 | At2g47470 | At3g43300 | At4g14480 | At5g04430 | At5g43830 |
| At1g04270 | At1g48850 | At2g19730 | At2g47510 | At3g44110 | At4g14880 | At5g04590 | At5g43940 |
| At1g04410 | At1g48920 | At2g19760 | At3g01120 | At3g44310 | At4g14960 | At5g05010 | At5g43960 |
| At1g04430 | At1g49670 | At2g19940 | At3g01280 | At3g44320 | At4g16155 | At5g05670 | At5g44130 |
| At1g04820 | At1g49760 | At2g20190 | At3g01340 | At3g44330 | At4g16450 | At5g05740 | At5g44320 |
| At1g05500 | At1g50250 | At2g20260 | At3g01500 | At3g45030 | At4g17100 | At5g06060 | At5g44340 |
| At1g05805 | At1g50480 | At2g20360 | At3g01670 | At3g45190 | At4g17390 | At5g06290 | At5g46070 |
| At1g06290 | At1g50700 | At2g20580 | At3g01680 | At3g45780 | At4g17520 | At5g06530 | At5g46110 |
| At1g06550 | At1g51410 | At2g20610 | At3g01780 | At3g45980 | At4g17600 | At5g07350 | At5g46290 |
| At1g06680 | At1g51980 | At2g20990 | At3g01910 | At3g46040 | At4g18030 | At5g07440 | At5g46750 |
| At1g06680 | At1g52280 | At2g21060 | At3g02090 | At3g46060 | At4g18290 | At5g07460 | At5g46800 |
| At1g06950 | At1g52400 | At2g21170 | At3g02360 | At3g46740 | At4g18430 | At5g07470 | At5g47110 |
| At1g07350 | At1g52570 | At2g21330 | At3g02720 | At3g46780 | At4g18480 | At5g08280 | At5g47210 |
| At1g07810 | At1g53210 | At2g21390 | At3g02880 | At3g46970 | At4g18820 | At5g08300 | At5g47650 |
| At1g07890 | At1g53500 | At2g21410 | At3g03250 | At3g47370 | At4g19710 | At5g08450 | At5g47690 |
| At1g07940 | At1g54040 | At2g21530 | At3g03780 | At3g47470 | At4g20110 | At5g08530 | At5g48180 |
| At1g08110 | At1g54270 | At2g21590 | At3g03960 | At3g47520 | At4g20360 | At5g08680 | At5g48230 |
| At1g08135 | At1g54410 | At2g21630 | At3g04790 | At3g47650 | At4g20890 | At5g09300 | At5g48300 |
| At1g08200 | At1g55480 | At2g21660 | At3g04840 | At3g47950 | At4g21710 | At5g09590 | At5g48375 |
| At1g08450 | At1g55510 | At2g21880 | At3g05280 | At3g48000 | At4g22240 | At5g09660 | At5g48440 |
| At1g08470 | At1g55670 | At2g22125 | At3g05420 | At3g48560 | At4g23630 | At5g09810 | At5g48480 |
| At1g08480 | At1g56070 | At2g22230 | At3g05530 | At3g48680 | At4g23650 | At5g09880 | At5g49460 |
| At1g08550 | At1g56190 | At2g22250 | At3g06050 | At3g48720 | At4g23670 | At5g10360 | At5g49720 |
| At1g08830 | At1g56340 | At2g22480 | At3g06510 | At3g48730 | At4g23730 | At5g10470 | At5g49910 |
| At1g09210 | At1g57660 | At2g23350 | At3g06580 | At3g48870 | At4g23850 | At5g10540 | At5g50000 |
| At1g09340 | At1g58380 | At2g24200 | At3g06670 | At3g48990 | At4g24190 | At5g10730 | At5g50250 |
| At1g09620 | At1g59870 | At2g24270 | At3g06810 | At3g49010 | At4g24280 | At5g10920 | At5g50850 |
| At1g09780 | At1g59900 | At2g25450 | At3g06850 | At3g49430 | At4g24510 | At5g11200 | At5g50920 |
| At1g10290 | At1g60200 | At2g25670 | At3g06860 | At3g49470 | At4g24620 | At5g11490 | At5g50950 |
| At1g10760 | At1g60810 | At2g25970 | At3g07110 | At3g49680 | At4g24680 | At5g11520 | At5g51820 |
| At1g10950 | At1g61520 | At2g26040 | At3g07390 | At3g49720 | At4g24770 | At5g11560 | At5g52310 |
| At1g11260 | At1g62020 | At2g26080 | At3g07770 | At3g50670 | At4g24830 | At5g11670 | At5g52470 |
| At1g11580 | At1g62560 | At2g26250 | At3g08510 | At3g50820 | At4g25130 | At5g11680 | At5g52520 |
| At1g11650 | At1g62750 | At2g27100 | At3g08580 | At3g51160 | At4g26630 | At5g11710 | At5g52560 |
| At1g11840 | At1g63000 | At2g27210 | At3g08680 | At3g51880 | At4g26870 | At5g11770 | At5g53460 |
| At1g11860 | At1g63180 | At2g27860 | At3g08940 | At3g52150 | At4g26900 | At5g12250 | At5g54190 |
| At1g11910 | At1g63500 | At2g28000 | At3g09440 | At3g52880 | At4g26910 | At5g13420 | At5g54270 |
| At1g12050 | At1g63770 | At2g28190 | At3g09630 | At3g52930 | At4g26970 | At5g13440 | At5g54500 |
| At1g12240 | At1g63940 | At2g29360 | At3g09790 | At3g52960 | At4g27000 | At5g13490 | At5g54810 |

| At1g12900 | At1g64190 | At2g29550 | At3g09820 | At3g52990 | At4g27230 | At5g13850 | At5g54900 |
|---|---|---|---|---|---|---|---|
| At1g13020 | At1g65440 | At2g30110 | At3g10920 | At3g53420 | At4g28750 | At5g13980 | At5g55220 |
| At1g13060 | At1g65860 | At2g30620 | At3g11130 | At3g53460 | At4g28770 | At5g14030 | At5g55660 |
| At1g13280 | At1g65930 | At2g30790 | At3g11130 | At3g53580 | At4g28910 | At5g14040 | At5g56010 |
| At1g13440 | At1g66200 | At2g30950 | At3g11210 | At3g53720 | At4g29010 | At5g14220 | At5g56260 |
| At1g13930 | At1g67090 | At2g30970 | At3g11400 | At3g54050 | At4g29060 | At5g14740 | At5g56630 |
| At1g14810 | At1g67230 | At2g31083 | At3g11630 | At3g54470 | At4g29120 | At5g14780 | At5g56680 |
| At1g14830 | At1g67730 | At2g31390 | At3g11910 | At3g54560 | At4g29220 | At5g15090 | At5g57290 |
| At1g15690 | At1g68020 | At2g31570 | At3g12110 | At3g54660 | At4g29260 | At5g15350 | At5g57870 |
| At1g15750 | At1g68530 | At2g32730 | At3g12290 | At3g54760 | At4g29350 | At5g15650 | At5g58140 |
| At1g15820 | At1g68560 | At2g32920 | At3g12390 | At3g54960 | At4g29410 | At5g16450 | At5g58330 |
| At1g16610 | At1g69620 | At2g33150 | At3g12580 | At3g55010 | At4g29680 | At5g16590 | At5g58420 |
| At1g16820 | At1g69830 | At2g33380 | At3g13300 | At3g55120 | At4g29840 | At5g16780 | At5g58440 |
| At1g17290 | At1g70310 | At2g33410 | At3g13470 | At3g55360 | At4g30190 | At5g16840 | At5g59350 |
| At1g17840 | At1g70410 | At2g33800 | At3g13860 | At3g55410 | At4g30530 | At5g16990 | At5g59910 |
| At1g18080 | At1g70490 | At2g34420 | At3g13870 | At3g55440 | At4g30910 | At5g17050 | At5g60160 |
| At1g18210 | At1g70730 | At2g34430 | At3g13930 | At3g55800 | At4g30920 | At5g17380 | At5g60660 |
| At1g18450 | At1g70770 | At2g34590 | At3g14067 | At3g56150 | At4g31180 | At5g17530 | At5g60980 |
| At1g19570 | At1g71500 | At2g35010 | At3g14100 | At3g56240 | At4g31490 | At5g17770 | At5g61780 |
| At1g19920 | At1g71695 | At2g35040 | At3g14415 | At3g56940 | At4g31990 | At5g17920 | At5g61790 |
| At1g20010 | At1g72370 | At2g35410 | At3g14420 | At3g57410 | At4g32520 | At5g19220 | At5g62200 |
| At1g20020 | At1g73600 | At2g35490 | At3g14990 | At3g57490 | At4g33010 | At5g19390 | At5g62530 |
| At1g20340 | At1g74260 | At2g35810 | At3g15020 | At3g57610 | At4g33030 | At5g19440 | At5g62670 |
| At1g20620 | At1g74910 | At2g35920 | At3g15060 | At3g58610 | At4g33090 | At5g19510 | At5g62690 |
| At1g20960 | At1g74960 | At2g36130 | At3g15360 | At3g58990 | At4g33510 | At5g19550 | At5g63310 |
| At1g21750 | At1g75780 | At2g36160 | At3g15520 | At3g59760 | At4g33680 | At5g19760 | At5g63840 |
| At1g22710 | At1g76030 | At2g36250 | At3g15730 | At3g59970 | At4g34110 | At5g19770 | At5g63860 |
| At1g23190 | At1g76100 | At2g36290 | At3g15980 | At3g60230 | At4g34200 | At5g20010 | At5g63890 |
| At1g23290 | At1g76150 | At2g36380 | At3g16110 | At3g60750 | At4g34450 | At5g20290 | At5g64350 |
| At1g23310 | At1g76180 | At2g36390 | At3g16400 | At3g60770 | At4g34870 | At5g20350 | At5g64860 |
| At1g23310 | At1g76680 | At2g36460 | At3g16640 | At3g61440 | At4g35100 | At5g20720 | At5g65750 |
| At1g23410 | At1g76810 | At2g36530 | At3g17390 | At3g61870 | At4g35260 | At5g23020 | At5g65760 |
| At1g23820 | At1g76880 | At2g36580 | At3g17810 | At3g62030 | At4g35470 | At5g23060 | At5g65770 |
| At1g24010 | At1g77060 | At2g36620 | At3g17970 | At3g62360 | At4g35630 | At5g23120 | At5g66040 |
| At1g24180 | At1g77180 | At2g36830 | At3g18035 | At3g62700 | At4g35790 | At5g23860 | At5g66190 |
| At1g24360 | At1g78370 | At2g36880 | At3g18780 | At3g63140 | At4g35800 | At5g23890 | At5g66510 |
| At1g24706 | At1g78570 | At2g37190 | At3g18820 | At3g63410 | At4g35830 | At5g24300 | At5g66570 |
| At1g26630 | At1g78860 | At2g37220 | At3g18890 | At3g63460 | At4g36020 | At5g26000 | At5g66680 |
| At1g26850 | At1g78880 | At2g37550 | At3g19010 | At3g63490 | At4g36130 | At5g26210 | At5g66720 |
| At1g27090 | At1g78900 | At2g37970 | At3g19170 | At3g63520 | At4g36195 | At5g26280 | At5g66760 |
| At1g27680 | At1g79040 | At2g38280 | At3g19760 | At4g00360 | At4g36250 | At5g26570 | At5g67030 |
| At1g27970 | At1g79230 | At2g38290 | At3g19820 | At4g00400 | At4g36690 | At5g26742 | At5g67500 |
| At1g29350 | At1g79440 | At2g38670 | At3g19930 | At4g01310 | At4g37300 | At5g26860 | AtCg00020 |
| At1g29880 | At1g79550 | At2g39730 | At3g20320 | At4g01370 | At4g37870 | At5g27380 | AtCg00120 |
| At1g29900 | At1g79920 | At2g39800 | At3g20920 | At4g02510 | At4g37910 | At5g27540 | AtCg00130 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| At1g29910 | At1g79930 | At2g39850 | At3g21560 | At4g02580 | At4g37930 | At5g27640 | AtCg00270 |
| At1g30230 | At1g79990 | At2g39990 | At3g22200 | At4g02770 | At4g38130 | At5g27700 | AtCg00280 |
| At1g30360 | At1g80300 | At2g40100 | At3g22330 | At4g02930 | At4g38350 | At5g28500 | AtCg00340 |
| At1g30380 | At1g80410 | At2g40490 | At3g22890 | At4g03200 | At4g38510 | At5g28540 | AtCg00350 |
| At1g30400 | At1g80480 | At2g40510 | At3g22960 | At4g03280 | At4g38600 | At5g28840 | AtCg00480 |
| At1g30510 | At1g80660 | At2g40840 | At3g23400 | At4g03520 | At4g38680 | At5g30510 | AtCg00490 |
| At1g30580 | At1g80930 | At2g41220 | At3g23700 | At4g04020 | At4g38740 | At5g34850 | AtCg00500 |
| At1g31190 | At2g01140 | At2g41530 | At3g23810 | At4g05020 | At4g38780 | At5g35160 | AtCg00540 |
| At1g31230 | At2g01720 | At2g41560 | At3g23840 | At4g08780 | At4g38800 | At5g35360 | AtCG00580 |
| At1g31330 | At2g02160 | At2g41740 | At3g23940 | At4g09010 | At4g38970 | At5g35590 | AtCg00670 |
| At1g32060 | At2g04030 | At2g41840 | At3g24170 | At4g09320 | At4g39080 | At5g35630 | AtCg00680 |
| At1g32470 | At2g05100 | At2g42500 | At3g24503 | At4g10320 | At4g39090 | At5g35790 | AtCg00720 |
| At1g33140 | At2g05710 | At2g42600 | At3g25150 | At4g10340 | At4g39210 | At5g36210 | AtCg00750 |
| At1g34130 | At2g05830 | At2g43030 | At3g25920 | At4g10960 | At4g39260 | At5g37360 | AtCg00800 |
| At1g35680 | At2g05990 | At2g43090 | At3g26340 | At4g11010 | At4g39330 | At5g37510 | AtCg00830 |
| At1g36160 | At2g06050 | At2g43100 | At3g26400 | At4g11220 | At4g39660 | At5g37600 | AtCg01060 |
| At1g36160 | At2g07050 | At2g43750 | At3g26520 | At4g11420 | At4g39680 | At5g38420 | AtMg00070 |
| At1g42550 | At2g07360 | At2g43910 | At3g26650 | At4g11820 | At4g39730 | At5g38470 | AtMg00160 |
| At1g42970 | At2g07785 | At2g44050 | At3g26710 | At4g12420 | At5g01530 | At5g38530 | AtMg01190 |
| **f. 974 proteins were identified from the 2nd MudPIT using the Protein Pilot algorithm.** | | | | | | | |
| At1g01090 | At1g30230 | At2g02160 | At2g45790 | At3g28530 | At4g12800 | At5g02960 | At5g41670 |
| At1g01510 | At1g30360 | At2g03440 | At2g45960 | At3g28730 | At4g12980 | At5g03040 | At5g41970 |
| At1g01610 | At1g30380 | At2g04030 | At2g46070 | At3g29075 | At4g13360 | At5g03300 | At5g42020 |
| At1g02080 | At1g30580 | At2g05100 | At2g46280 | At3g29320 | At4g13430 | At5g03630 | At5g42270 |
| At1g02280 | At1g31190 | At2g05710 | At2g46800 | At3g29360 | At4g13770 | At5g04140 | At5g42650 |
| At1g02560 | At1g31230 | At2g05830 | At2g47110 | At3g42170 | At4g13850 | At5g04280 | At5g42790 |
| At1g02780 | At1g31330 | At2g05990 | At2g47240 | At3g43300 | At4g13930 | At5g04360 | At5g43330 |
| At1g02840 | At1g32060 | At2g06050 | At2g47390 | At3g44110 | At4g13940 | At5g04430 | At5g43830 |
| At1g03090 | At1g32470 | At2g07050 | At2g47470 | At3g44310 | At4g14030 | At5g04590 | At5g43940 |
| At1g03220 | At1g33140 | At2g13560 | At2g47510 | At3g44330 | At4g14040 | At5g05010 | At5g43960 |
| At1g03475 | At1g34130 | At2g14170 | At2g47710 | At3g45030 | At4g14070 | At5g05670 | At5g44020 |
| At1g03680 | At1g36160 | At2g14720 | At3g01120 | At3g45140 | At4g14160 | At5g05740 | At5g44130 |
| At1g04080 | At1g42550 | At2g15620 | At3g01280 | At3g45190 | At4g14360 | At5g06060 | At5g44320 |
| At1g04170 | At1g42970 | At2g16600 | At3g01340 | At3g45780 | At4g14440 | At5g06290 | At5g44340 |
| At1g04270 | At1g43170 | At2g16940 | At3g01480 | At3g45980 | At4g14480 | At5g06530 | At5g45930 |
| At1g04410 | At1g43670 | At2g17420 | At3g01500 | At3g46040 | At4g14880 | At5g06600 | At5g46070 |
| At1g04710 | At1g44575 | At2g18020 | At3g01680 | At3g46060 | At4g14960 | At5g07020 | At5g46110 |
| At1g04850 | At1g44910 | At2g18960 | At3g01780 | At3g46740 | At4g15900 | At5g07090 | At5g46290 |
| At1g05190 | At1g45201 | At2g19520 | At3g01910 | At3g46780 | At4g16130 | At5g07350 | At5g46750 |
| At1g05500 | At1g47128 | At2g19730 | At3g02090 | At3g46970 | At4g16155 | At5g07440 | At5g46800 |
| At1g05805 | At1g47260 | At2g19760 | At3g02360 | At3g47370 | At4g17100 | At5g07460 | At5g47030 |
| At1g05810 | At1g47420 | At2g19940 | At3g02420 | At3g47470 | At4g17170 | At5g07470 | At5g47110 |
| At1g06290 | At1g48030 | At2g20190 | At3g02720 | At3g47520 | At4g17390 | At5g08300 | At5g47210 |
| At1g06550 | At1g48410 | At2g20260 | At3g02770 | At3g47650 | At4g18030 | At5g08450 | At5g47650 |
| At1g06680 | At1g48600 | At2g20360 | At3g02870 | At3g48000 | At4g18290 | At5g08530 | At5g47690 |

| At1g06840 | At1g48605 | At2g20580 | At3g02880 | At3g48560 | At4g18820 | At5g08680 | At5g47930 |
|---|---|---|---|---|---|---|---|
| At1g06950 | At1g48620 | At2g20610 | At3g03100 | At3g48690 | At4g20360 | At5g09590 | At5g48180 |
| At1g07320 | At1g48850 | At2g20630 | At3g03250 | At3g48720 | At4g21150 | At5g09810 | At5g48230 |
| At1g07370 | At1g48920 | At2g20990 | At3g03780 | At3g48730 | At4g21710 | At5g09880 | At5g48300 |
| At1g07750 | At1g49240 | At2g21060 | At3g03960 | At3g48990 | At4g21860 | At5g10010 | At5g48375 |
| At1g07810 | At1g49670 | At2g21160 | At3g04120 | At3g49010 | At4g22240 | At5g10360 | At5g48440 |
| At1g07890 | At1g49760 | At2g21170 | At3g04790 | At3g49120 | At4g23630 | At5g10470 | At5g48480 |
| At1g07930 | At1g50010 | At2g21330 | At3g05420 | At3g49470 | At4g23650 | At5g10540 | At5g49460 |
| At1g08135 | At1g50200 | At2g21390 | At3g06050 | At3g49600 | At4g23670 | At5g10730 | At5g49720 |
| At1g08200 | At1g50250 | At2g21530 | At3g06510 | At3g49680 | At4g23730 | At5g10920 | At5g49810 |
| At1g08420 | At1g50380 | At2g21590 | At3g06580 | At3g50670 | At4g23850 | At5g11170 | At5g49910 |
| At1g08470 | At1g50480 | At2g21660 | At3g06810 | At3g50820 | At4g24190 | At5g11490 | At5g50000 |
| At1g08480 | At1g51710 | At2g21880 | At3g06860 | At3g51840 | At4g24280 | At5g11560 | At5g50250 |
| At1g08550 | At1g51980 | At2g22125 | At3g07100 | At3g51880 | At4g24510 | At5g11670 | At5g50850 |
| At1g08830 | At1g52280 | At2g22230 | At3g07110 | At3g52140 | At4g24620 | At5g11680 | At5g50920 |
| At1g09100 | At1g52380 | At2g22480 | At3g07390 | At3g52150 | At4g24770 | At5g11710 | At5g50950 |
| At1g09210 | At1g52400 | At2g23350 | At3g07770 | At3g52230 | At4g25130 | At5g11770 | At5g51820 |
| At1g09340 | At1g53210 | At2g24200 | At3g08510 | At3g52880 | At4g25630 | At5g12040 | At5g52310 |
| At1g09620 | At1g53240 | At2g24270 | At3g08530 | At3g52930 | At4g26270 | At5g12250 | At5g52520 |
| At1g09780 | At1g53500 | At2g24590 | At3g08580 | At3g52960 | At4g26630 | At5g12860 | At5g52560 |
| At1g10630 | At1g54040 | At2g24820 | At3g08590 | At3g52990 | At4g26840 | At5g13420 | At5g53320 |
| At1g10670 | At1g54270 | At2g25450 | At3g08680 | At3g53110 | At4g26900 | At5g13430 | At5g53460 |
| At1g10770 | At1g54410 | At2g25970 | At3g08940 | At3g53420 | At4g26970 | At5g13520 | At5g54270 |
| At1g10950 | At1g55480 | At2g26040 | At3g09440 | At3g53430 | At4g27000 | At5g14030 | At5g54500 |
| At1g11260 | At1g55510 | At2g26080 | At3g09630 | At3g53520 | At4g27440 | At5g14040 | At5g54810 |
| At1g11410 | At1g55670 | At2g26250 | At3g09820 | At3g53580 | At4g27500 | At5g14220 | At5g54900 |
| At1g11580 | At1g56070 | At2g27020 | At3g09840 | At3g53720 | At4g27560 | At5g14740 | At5g55070 |
| At1g11750 | At1g56190 | At2g27100 | At3g10370 | At3g53890 | At4g28650 | At5g14780 | At5g55220 |
| At1g11840 | At1g56340 | At2g27720 | At3g10410 | At3g54050 | At4g28750 | At5g15090 | At5g55660 |
| At1g11860 | At1g56580 | At2g28000 | At3g10920 | At3g54110 | At4g28770 | At5g15350 | At5g56010 |
| At1g11910 | At1g57660 | At2g28190 | At3g11210 | At3g54470 | At4g28910 | At5g15650 | At5g56260 |
| At1g12050 | At1g57720 | At2g29360 | At3g11400 | At3g54660 | At4g29010 | At5g16590 | At5g56500 |
| At1g12240 | At1g59610 | At2g29550 | At3g11510 | At3g54760 | At4g29060 | At5g16840 | At5g56680 |
| At1g12250 | At1g59870 | At2g30110 | At3g11630 | At3g54960 | At4g29120 | At5g16990 | At5g57110 |
| At1g12900 | At1g59900 | At2g30490 | At3g11830 | At3g55010 | At4g29220 | At5g17050 | At5g57290 |
| At1g13020 | At1g60200 | At2g30620 | At3g12290 | At3g55120 | At4g29260 | At5g17170 | At5g57350 |
| At1g13060 | At1g61520 | At2g30950 | At3g12390 | At3g55360 | At4g29350 | At5g17380 | At5g57460 |
| At1g13080 | At1g62560 | At2g30970 | At3g12580 | At3g55410 | At4g29410 | At5g17530 | At5g57870 |
| At1g13280 | At1g62740 | At2g31390 | At3g12810 | At3g55440 | At4g29680 | At5g17710 | At5g58140 |
| At1g13440 | At1g62750 | At2g31570 | At3g12860 | At3g55800 | At4g29840 | At5g17770 | At5g58340 |
| At1g13930 | At1g63000 | At2g32730 | At3g13235 | At3g56150 | At4g30160 | At5g17920 | At5g58440 |
| At1g14820 | At1g63180 | At2g32920 | At3g13300 | At3g56240 | At4g30190 | At5g18380 | At5g59370 |
| At1g14830 | At1g63500 | At2g33150 | At3g13470 | At3g56340 | At4g30530 | At5g19390 | At5g59420 |
| At1g14980 | At1g63770 | At2g33380 | At3g13772 | At3g57020 | At4g31180 | At5g19440 | At5g60160 |
| At1g15290 | At1g63940 | At2g33410 | At3g13860 | At3g57330 | At4g31300 | At5g19510 | At5g60390 |

| At1g15340 | At1g64190 | At2g33800 | At3g13870 | At3g57610 | At4g31490 | At5g19550 | At5g60540 |
|---|---|---|---|---|---|---|---|
| At1g15500 | At1g64370 | At2g34430 | At3g13930 | At3g58610 | At4g31580 | At5g19690 | At5g60600 |
| At1g15690 | At1g65440 | At2g34660 | At3g14067 | At3g58990 | At4g31700 | At5g19760 | At5g60980 |
| At1g15750 | At1g65930 | At2g35010 | At3g14100 | At3g59020 | At4g31860 | At5g19770 | At5g61780 |
| At1g15820 | At1g67090 | At2g35040 | At3g14415 | At3g59760 | At4g31880 | At5g20010 | At5g61790 |
| At1g16610 | At1g67230 | At2g35410 | At3g14990 | At3g59920 | At4g31990 | At5g20290 | At5g62200 |
| At1g16720 | At1g67730 | At2g35490 | At3g15090 | At3g59970 | At4g32770 | At5g20350 | At5g62530 |
| At1g17220 | At1g68010 | At2g35810 | At3g15360 | At3g60240 | At4g33010 | At5g20720 | At5g62670 |
| At1g17290 | At1g68020 | At2g35840 | At3g15520 | At3g60245 | At4g33030 | At5g23020 | At5g62690 |
| At1g17840 | At1g68530 | At2g35920 | At3g15730 | At3g60340 | At4g33090 | At5g23060 | At5g62790 |
| At1g18070 | At1g68560 | At2g36130 | At3g16110 | At3g60600 | At4g33510 | At5g23120 | At5g63310 |
| At1g18080 | At1g69830 | At2g36290 | At3g16400 | At3g60750 | At4g33680 | At5g23860 | At5g63510 |
| At1g18210 | At1g70310 | At2g36380 | At3g16640 | At3g61440 | At4g34110 | At5g24300 | At5g63550 |
| At1g18440 | At1g70410 | At2g36390 | At3g17070 | At3g62030 | At4g34200 | At5g25980 | At5g63620 |
| At1g18540 | At1g70600 | At2g36460 | At3g17390 | At3g62110 | At4g34450 | At5g26000 | At5g63840 |
| At1g19570 | At1g70730 | At2g36530 | At3g17810 | At3g62360 | At4g34670 | At5g26210 | At5g63890 |
| At1g19580 | At1g70770 | At2g36620 | At3g17970 | At3g62700 | At4g35100 | At5g26280 | At5g64350 |
| At1g19870 | At1g71220 | At2g36830 | At3g18035 | At3g62830 | At4g35260 | At5g26570 | At5g64860 |
| At1g19920 | At1g71310 | At2g36880 | At3g18820 | At3g62870 | At4g35450 | At5g26742 | At5g65750 |
| At1g20010 | At1g71500 | At2g37220 | At3g18860 | At3g63140 | At4g35470 | At5g26830 | At5g65760 |
| At1g20020 | At1g71695 | At2g38040 | At3g18890 | At3g63410 | At4g35630 | At5g26860 | At5g65770 |
| At1g20330 | At1g72370 | At2g38230 | At3g19010 | At3g63460 | At4g35790 | At5g27380 | At5g66040 |
| At1g20340 | At1g73600 | At2g38280 | At3g19170 | At3g63490 | At4g35800 | At5g27430 | At5g66190 |
| At1g20620 | At1g74960 | At2g38290 | At3g19760 | At3g63520 | At4g35830 | At5g27540 | At5g66510 |
| At1g20960 | At1g75220 | At2g38670 | At3g19820 | At4g00100 | At4g36020 | At5g27640 | At5g66530 |
| At1g21400 | At1g76030 | At2g39730 | At3g19930 | At4g00360 | At4g36130 | At5g27670 | At5g66570 |
| At1g21540 | At1g76100 | At2g39800 | At3g20320 | At4g00570 | At4g36195 | At5g27700 | At5g66680 |
| At1g21670 | At1g76180 | At2g39850 | At3g20920 | At4g00630 | At4g36250 | At5g28500 | At5g66720 |
| At1g21750 | At1g76550 | At2g39990 | At3g22200 | At4g01850 | At4g36690 | At5g28540 | At5g66760 |
| At1g22410 | At1g76680 | At2g40060 | At3g22330 | At4g02080 | At4g37300 | At5g28840 | At5g67030 |
| At1g22710 | At1g76880 | At2g40100 | At3g22890 | At4g02450 | At4g37870 | At5g30510 | At5g67500 |
| At1g23190 | At1g77060 | At2g40490 | At3g22960 | At4g02510 | At4g37910 | At5g34850 | At5g67590 |
| At1g23310 | At1g77180 | At2g40840 | At3g23400 | At4g02580 | At4g37930 | At5g35160 | AtCg00120 |
| At1g23740 | At1g77590 | At2g41530 | At3g23700 | At4g02770 | At4g38130 | At5g35360 | AtCg00130 |
| At1g23820 | At1g78370 | At2g41740 | At3g23810 | At4g02930 | At4g38350 | At5g35590 | AtCG00270 |
| At1g24010 | At1g78850 | At2g41840 | At3g23840 | At4g03200 | At4g38510 | At5g35630 | AtCG00280 |
| At1g24180 | At1g78900 | At2g42500 | At3g24170 | At4g03280 | At4g38680 | At5g35790 | AtCg00340 |
| At1g24360 | At1g79040 | At2g42520 | At3g25230 | At4g03520 | At4g38740 | At5g36210 | AtCg00350 |
| At1g24706 | At1g79210 | At2g42600 | At3g25860 | At4g04020 | At4g38800 | At5g36700 | AtCg00480 |
| At1g26110 | At1g79230 | At2g43030 | At3g25920 | At4g04950 | At4g38970 | At5g36880 | AtCg00490 |
| At1g26630 | At1g79440 | At2g43100 | At3g26060 | At4g05020 | At4g39080 | At5g37360 | AtCg00500 |
| At1g26850 | At1g79550 | At2g43750 | At3g26400 | At4g05590 | At4g39090 | At5g37510 | AtCg00540 |
| At1g26880 | At1g79930 | At2g44050 | At3g26430 | At4g09010 | At4g39260 | At5g37600 | AtCG00580 |
| At1g26910 | At1g79990 | At2g44060 | At3g26520 | At4g09320 | At4g39330 | At5g38410 | AtCg00670 |
| At1g27090 | At1g80030 | At2g44100 | At3g26650 | At4g10320 | At4g39660 | At5g38470 | AtCg00680 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| At1g27770 | At1g80070 | At2g44160 | At3g26710 | At4g10340 | At4g39680 | At5g38660 | AtCg00730 |
| At1g27970 | At1g80300 | At2g44490 | At3g26720 | At4g11010 | At4g39730 | At5g39410 | AtCg00830 |
| At1g29350 | At1g80460 | At2g44920 | At3g26740 | At4g11220 | At4g39860 | At5g39570 | AtMg00070 |
| At1g29880 | At1g80480 | At2g45300 | At3g26744 | At4g11420 | At5g01410 | At5g40340 | AtMg00160 |
| At1g29900 | At1g80930 | At2g45440 | At3g26935 | At4g11600 | At5g01530 | At5g40370 | AtMg01190 |
| At1g29910 | At2g01140 | At2g45540 | At3g27300 | At4g11820 | At5g02240 | At5g40490 | |
| At1g30120 | At2g01720 | At2g45600 | At3g27380 | At4g12420 | At5g02500 | At5g40810 | |

**Table 2-2. Twenty-eight proteins were identified in multiple spots in the gel-based methods.**

| AGI | BR (Spot #) | NR (Spot #) |
|---|---|---|
| At1g06680 | 32,127,130,131 | 3F12, SB2, 4C9 |
| At1g29930 | 20,116 | |
| At1g67090 | 40, 44 | 6D5, 6F10 |
| At1g78900 | 11, 58, | |
| At2g21660 | | 3A7, 4C6 |
| At2g28190 | | 3A9, 4C7 |
| At2g32240 | | 4E1, 6F9 |
| At2g35490 | | 1A7, 6H10 |
| At2g39730 | 15, 27, 88 | 3C4, 6A1, 6F11 |
| At2g43750 | 99, 100 | |
| At2g46320 | | 4F12, 5B6, 7A1 |
| At3g01500 | 128, 129 | |
| At3g04120 | 46, 97 | |
| At3g50820 | | 5E9, 6C6,6C7,6C8 |
| At3g52960 | | 5D4, 6B7 |
| At4g24280 | | 5G8, 6G2 |
| At4g39730 | | 1B1, 6G6, |
| At5g08670 | | 4F10, 5C1 |
| At5g08680 | | 4F10, 5C1 |
| At5g08690 | | 3D9, 3D10, 4F9, 4F10, 5C1, 5C6 |
| At5g26000 | 1, 2, 11, 12, 35, 36, 41, 51, 52, 54, 55, 56 | 1C2, 1C3, 1C4, 1F2, 1F10, 3E1, 3E10, 3E11, 3E12, 3E2, 3E4, 3E6, 3E9, 3D12, 3D3, 3F2, 3F3, 3F4, 4B6, 4B7, 4B9, 4B10, 4F6, 4F8, 4G8, 4G12, 4H1, 4H2, 4H6, 4H7, 4H9, 5B5, 5B8, 5B9, 5C9, 5C10, 7A12 |
| At5g35630 | 16, 77, 88 | |
| At5g44340 | 71, 72 | |
| At5g49910 | | 5G8, 6G2 |
| At5g66570 | | 5 E8, 6C6, 6C7, 6C8, 6F4, 6F5, 6F6, 6G12, 6G4, 6H4 |
| AtCg00120 | 14, 42, 76 | |
| AtCg00480 | 24, 122 | |
| AtCg00490 | 3, 4, 21, 37, 44, 69, 123, 134 | 1B10, 1B11, 1B12, 1B8, 1B9, 1F11, 3D6, 5D7, 1G12, 4G3, 4H8, 5A12, 6D5, 6F10, 6G7, 6H11 |

**Table 2-3. Guard cell proteins identified by any two proteomic methods.**

| **A. Nineteen proteins were identified from all three methods.** | | | | |
|---|---|---|---|---|
| **AGI** | **MW (kD)** | **pI** | **Predicted location** | **Name** |
| At1g06680 | 28.1 | 7.5 | chloroplast | a 23 kD extrinsic protein that is part of photosystem II and participates in the regulation of oxygen evolution |
| At1g21750 | 55.6 | 4.5 | secreted | protein disulfide isomerase |
| At1g51980 | 54.4 | 6.3 | undefined | mitochondrial processing peptidase alpha subunit |
| At1g53240 | 35.8 | 8.6 | undefined | malate dehydrogenase (NAD) |
| At1g56340 | 48.5 | 4.2 | secreted | calreticulin (Crt1) mRNA |
| At1g67090 | 20.2 | 7.8 | chloroplast | RuBisCO small subunit 1A (RBCS-1A) (ATS1A) |
| At2g21660 | 16.9 | 5.9 | undefined | small glycine-rich protein (GRPs) |
| At2g37220 | 30.7 | 4.8 | chloroplast | 29 kDa ribonucleoprotein |
| At2g39730 | 52.0 | 5.9 | chloroplast | Rubisco activase |
| At3g01500 | 29.5 | 5.5 | other (e.g. cytoplasm) | similar to carbonic anhydrase |
| At3g04120 | 36.9 | 7.1 | undefined | glyceraldehyde-3-phosphate dehydrogenase C subunit (GapC) |
| At3g50820 | 35.0 | 6 | chloroplast | encodes a protein which is an extrinsic subunit of photosystem II |
| At3g55800 | 42.4 | 6.5 | chloroplast | the chloroplast enzyme sedoheptulose-1 |
| At4g39260 | 16.6 | 5.3 | undefined | a glycine-rich protein with RNA binding domain at the N-terminus |
| At5g15090 | 29.2 | 8.7 | undefined | porin |
| At5g20720 | 26.8 | 9.4 | chloroplast | a chloroplast co-chaperonin with similarity to CPN21 from spinach |
| At5g26000 | 61.1 | 5.7 | secreted | member of Glycoside Hydrolase Family 1 |
| At5g66570 | 35.1 | 5.3 | chloroplast | mutant has High chlorophyll fluorescence |
| AtCg00490 | 53.0 | 6.2 | chloroplast | large subunit of RuBisCO |
| **B. Two proteins were identified from the two gel-based methods in addition to the 19 proteins** | | | | |
| AGI | MW (kD) | pI | Predicted location | Name |
| At1g29930 | 8.2 | 5.3 | chloroplast | subunit of light-harvesting complex II |
| At5g08690 | 59.7 | 6.6 | mitochondrion | ATP synthase beta chain 2 |
| **C. Thirty-two proteins were identified from BR and MudPIT methods in addition to the 19 proteins.** | | | | |
| AGI | MW (kD) | pI | Predicted location | Name |
| At1g07890 | 27.6 | 6 | other (e.g. cytoplasm) | similar to L-ascorbate peroxidase 1b (APX1b) |
| At1g09210 | 48.2 | 4.1 | secreted | calreticulin 2 (CRT2) |
| At1g09780 | 60.6 | 5.2 | other (e.g. cytoplasm) | 2,3-biphosphoglycerate-independent phosphoglycerate mutase |
| At1g20340 | 17.0 | 4.8 | chloroplast | recombination and DNA-damage resistance |

| | | | | |
|---|---|---|---|---|
| | | | | protein |
| At1g32060 | 44.5 | 5.9 | chloroplast | phosphoribulokinase (PRK) |
| At1g63940 | 52.5 | 7.5 | chloroplast | monodehydroascorbate reductase |
| At1g76030 | 54.1 | 4.7 | other (e.g. cytoplasm) | vacuolar ATP synthase subunit B |
| At1g78900 | 68.8 | 4.9 | other (e.g. cytoplasm) | similar to ATP synthase beta chain 2 |
| At2g20420 | 45.3 | 6.7 | mitochondrion | succinyl-CoA ligase (GDP-forming) beta-chain |
| At2g24200 | 54.5 | 5.7 | other (e.g. cytoplasm) | cytosol aminopeptidase |
| At2g28000 | 62.1 | 4.8 | chloroplast | chaperonin-60 alpha |
| At2g36530 | 47.7 | 5.5 | undefined | an enolase |
| At2g43750 | 41.7 | 8.2 | chloroplast | O-acetylserine (thiol) lyase (OAS-TL) isoform oasB |
| At3g08590 | 60.8 | 5.6 | other (e.g. cytoplasm) | 2,3-biphosphoglycerate-independent phosphoglycerate mutase |
| At3g11630 | 29.1 | 7.6 | chloroplast | 2-cys peroxiredoxin |
| At3g16640 | 18.9 | 4.3 | undefined | TCTP homolog |
| At3g18780 | 41.2 | 5.4 | other (e.g. cytoplasm) | actin 2 |
| At3g55440 | 27.2 | 5.2 | undefined | columbia cytosolic triose phosphate isomerase |
| At3g62030 | 28.2 | 8.8 | chloroplast | nuclear-encoded chloroplast stromal cyclophilin |
| At4g01850 | 43.3 | 5.9 | other (e.g. cytoplasm) | S-adenosylmethionine synthetase 2 |
| At4g02930 | 49.4 | 6.7 | undefined | elongation factor Tu |
| At4g14960 | 47.2 | 8.2 | undefined | an alpha-tubulin isoform required for right handed helical growth |
| At4g24770 | 35.8 | 4.3 | chloroplast | a chloroplast RNA-binding protein |
| At5g17920 | 84.4 | 6.5 | undefined | a protein predicted to have cobalamin-independent methionine synthase activity |
| At5g23120 | 44.1 | 7.5 | chloroplast | a stability and/or assembly factor of photosystem II |
| At5g27640 | 81.9 | 4.9 | undefined | a member of eukaryotic translation initiation factor 3B family |
| At5g35630 | 47.4 | 6.9 | chloroplast | chloroplastic glutamine synthetase |
| At5g38420 | 20.4 | 7.8 | chloroplast | RuBisCO small subunit 2B |
| At5g44340 | 49.8 | 4.5 | undefined | beta tubulin gene |
| AtCg00120 | 55.3 | 4.9 | chloroplast | ATPase alpha subunit |
| AtCg00480 | 53.9 | 5.2 | chloroplast | chloroplast-encoded gene for beta subunit of ATP synthase |
| AtMg01190 | 55.0 | 6.5 | mitochondrion | ATPase subunit 1 |
| **D. Twenty-three proteins were identified from NR and LC-MALDI MudPIT methods in addition of the 19 proteins.** | | | | |
| AGI | MW (kD) | pI | Predicted location | Name |
| At1g13440 | 36.9 | 7.2 | undefined | glyceraldehyde 3-phosphate dehydrogenase |
| At1g20010 | 50.3 | 4.4 | other (e.g. cytoplasm) | beta tubulin |

| | | | | |
|---|---|---|---|---|
| At1g54040 | 28.5 | 4.9 | undefined | epithiospecifier protein |
| At1g78370 | 25.0 | 5.5 | undefined | glutathione transferase belonging to the tau class of GSTs |
| At2g05990 | 41.2 | 9.4 | chloroplast | enoyl-ACP reductase a component of the fatty acid synthase complex |
| At2g21170 | 33.3 | 8 | chloroplast | triosephosphate isomerase |
| At2g21330 | 42.9 | 6.5 | chloroplast | similar to fructose-bisphosphate aldolase |
| At2g28190 | 22.2 | 7 | chloroplast | superoxide dismutase |
| At2g32240 | 86.2 | 4.5 | undefined | expressed protein |
| At2g35490 | 40.5 | 4.2 | chloroplast | plastid-lipid associated protein PAP |
| At2g43090 | 26.8 | 6.8 | chloroplast | aconitase C-terminal domain-containing protein |
| At2g43100 | 27.0 | 6 | chloroplast | aconitase C-terminal domain-containing protein |
| At3g15020 | 35.9 | 8.4 | undefined | malate dehydrogenase |
| At3g52960 | 24.7 | 9.6 | chloroplast | peroxiredoxin type 2 |
| At3g53460 | 36.0 | 5 | chloroplast | a nuclear gene with a consensus RNA-binding domain that is localized to the chloroplast |
| At3g58990 | 27.2 | 6.9 | chloroplast | aconitase C-terminal domain-containing protein |
| At4g24280 | 76.5 | 4.8 | chloroplast | heat shock protein 70 |
| At4g38970 | 43.0 | 7.3 | chloroplast | fructose-bisphosphate aldolase |
| At4g39730 | 20.1 | 4.8 | secreted | lipid-associated family protein |
| At5g08680 | 59.9 | 6.5 | undefined | ATP synthase beta chain |
| At5g48480 | 17.6 | 4.5 | other (e.g. cytoplasm) | expressed protein |
| At5g49910 | 77.0 | 4.9 | chloroplast | heat shock protein 70 (Hsc70-7) |
| At5g57290 | 11.9 | 4.2 | undefined | 60S acidic ribosomal protein P3 (RPP3B) |

The subcellular localizations are predicted by the software provided by TAIR.

**Table 2-4. Enrichment of GO categories was obtained by the topGO package.**

| **A. Enrichment of GO categories for Biological Process.** | | | |
|---|---|---|---|
| **Rank** | **GO. ID** | **Term** | **log10(1/P-value)** |
| 1 | GO:0009409 | response to cold | 23 |
| 2 | GO:0006412 | translation | 22 |
| 3 | GO:0006096 | glycolysis | 16 |
| 4 | GO:0015979 | photosynthesis | 14 |
| 5 | GO:0019684 | photosynthesis, light reaction | 13 |
| 6 | GO:0006457 | protein folding | 12 |
| 7 | GO:0006633 | fatty acid biosynthetic process | 10 |
| 8 | GO:0006520 | amino acid metabolic process | 10 |
| 9 | GO:0006996 | organelle organization and biogenesis | 10 |
| 10 | GO:0015977 | carbon utilization by fixation of carbon... | 10 |
| 11 | GO:0044262 | cellular carbohydrate metabolic process | 10 |
| 12 | GO:0006461 | protein complex assembly | 10 |
| 13 | GO:0006886 | intracellular protein transport | 10 |
| 14 | GO:0044249 | cellular biosynthetic process | 10 |
| 15 | GO:0006098 | pentose-phosphate shunt | 9 |
| 16 | GO:0009853 | photorespiration | 9 |
| 17 | GO:0043648 | dicarboxylic acid metabolic process | 9 |
| 18 | GO:0015995 | chlorophyll biosynthetic process | 9 |
| 19 | GO:0044248 | cellular catabolic process | 8 |
| 20 | GO:0016051 | carbohydrate biosynthetic process | 8 |
| 21 | GO:0000097 | sulfur amino acid biosynthetic process | 8 |
| 22 | GO:0015992 | proton transport | 8 |
| 23 | GO:0006084 | acetyl-CoA metabolic process | 8 |
| 24 | GO:0008652 | amino acid biosynthetic process | 8 |
| 25 | GO:0022613 | ribonucleoprotein complex biogenesis and... | 8 |
| 26 | GO:0019252 | starch biosynthetic process | 8 |
| 27 | GO:0009066 | aspartate family amino acid metabolic pr... | 8 |
| 28 | GO:0008152 | metabolic process | 8 |
| 29 | GO:0019318 | hexose metabolic process | 8 |
| 30 | GO:0006979 | response to oxidative stress | 7 |
| 31 | GO:0006164 | purine nucleotide biosynthetic process | 7 |
| 32 | GO:0006091 | generation of precursor metabolites and ... | 7 |
| 33 | GO:0009793 | embryonic development ending in seed dor... | 7 |
| 34 | GO:0009416 | response to light stimulus | 7 |
| 35 | GO:0006100 | tricarboxylic acid cycle intermediate me... | 7 |
| 36 | GO:0019321 | pentose metabolic process | 6 |
| 37 | GO:0009657 | plastid organization and biogenesis | 6 |
| 38 | GO:0010109 | regulation of photosynthesis | 6 |
| 39 | GO:0046483 | heterocycle metabolic process | 6 |
| 40 | GO:0006913 | nucleocytoplasmic transport | 6 |

| | | | |
|---|---|---|---|
| 41 | GO:0006090 | pyruvate metabolic process | 6 |
| 42 | GO:0016192 | vesicle-mediated transport | 6 |
| 43 | GO:0006414 | translational elongation | 6 |
| 44 | GO:0032787 | monocarboxylic acid metabolic process | 6 |
| **B. Enrichment of GO categories for Molecular Function** | | | |
| Rank | GO.ID | Term | log10(1/P-value) |
| 1 | GO:0003735 | structural constituent of ribosome | 18 |
| 2 | GO:0003824 | catalytic activity | 14 |
| 3 | GO:0016836 | hydro-lyase activity | 14 |
| 4 | GO:0016168 | chlorophyll binding | 14 |
| 5 | GO:0016853 | isomerase activity | 12 |
| 6 | GO:0008266 | poly(U) binding | 11 |
| 7 | GO:0016624 | oxidoreductase activity, acting on the a... | 11 |
| 8 | GO:0005200 | structural constituent of cytoskeleton | 10 |
| 9 | GO:0009055 | electron carrier activity | 8 |
| 10 | GO:0016616 | oxidoreductase activity, acting on the C... | 8 |
| 11 | GO:0016831 | carboxy-lyase activity | 7 |
| 12 | GO:0016615 | malate dehydrogenase activity | 7 |
| 13 | GO:0003746 | translation elongation factor activity | 7 |
| 14 | GO:0050662 | coenzyme binding | 7 |
| 15 | GO:0016417 | S-acyltransferase activity | 7 |
| 16 | GO:0008943 | glyceraldehyde-3-phosphate dehydrogenase... | 7 |
| 17 | GO:0008237 | metallopeptidase activity | 7 |
| 18 | GO:0016627 | oxidoreductase activity, acting on the C... | 7 |
| 19 | GO:0004028 | 3-chloroallyl aldehyde dehydrogenase act... | 7 |
| 20 | GO:0008483 | transaminase activity | 7 |
| 21 | GO:0015078 | hydrogen ion transmembrane transporter a... | 7 |
| 22 | GO:0016491 | oxidoreductase activity | 7 |
| 23 | GO:0004576 | oligosaccharyl transferase activity | 7 |
| 24 | GO:0000166 | nucleotide binding | 7 |
| 25 | GO:0015662 | ATPase activity, coupled to transmembran... | 7 |
| 26 | GO:0016887 | ATPase activity | 6 |
| 27 | GO:0016667 | oxidoreductase activity, acting on sulfu... | 6 |
| 28 | GO:0004177 | aminopeptidase activity | 6 |
| 29 | GO:0016857 | racemase and epimerase activity, acting ... | 6 |
| **C. Enrichment of GO categories for Cellular Component** | | | |
| Rank | GO.ID | Term | log10(1/P-value) |
| 1 | GO:0009535 | chloroplast thylakoid membrane | 30 |
| 2 | GO:0005739 | mitochondrion | 30 |
| 3 | GO:0010287 | plastoglobule | 28 |
| 4 | GO:0043234 | protein complex | 23 |
| 5 | GO:0044444 | cytoplasmic part | 20 |
| 6 | GO:0005783 | endoplasmic reticulum | 19 |
| 7 | GO:0010319 | stromule | 18 |

| | | | |
|---|---|---|---|
| 8 | GO:0005737 | cytoplasm | 17 |
| 9 | GO:0005829 | cytosol | 15 |
| 10 | GO:0044446 | intracellular organelle part | 13 |
| 11 | GO:0031090 | organelle membrane | 12 |
| 12 | GO:0009570 | chloroplast stroma | 12 |
| 13 | GO:0009536 | plastid | 11 |
| 14 | GO:0022627 | cytosolic small ribosomal subunit | 10 |
| 15 | GO:0005794 | golgi apparatus | 10 |
| 16 | GO:0031967 | organelle envelope | 10 |
| 17 | GO:0030076 | light-harvesting complex | 10 |
| 18 | GO:0045298 | tubulin complex | 9 |
| 19 | GO:0022626 | cytosolic ribosome | 8 |
| 20 | GO:0044445 | cytosolic part | 8 |
| 21 | GO:0031966 | mitochondrial membrane | 7 |
| 22 | GO:0009507 | chloroplast | 7 |
| 23 | GO:0009523 | photosystem II | 7 |
| 24 | GO:0005773 | vacuole | 7 |
| 25 | GO:0043233 | organelle lumen | 6 |
| 26 | GO:0030126 | COPI vesicle coat | 6 |

**Table 2-5. Identified guard cell signaling proteins in our guard cell proteome.**

| **A. Twelve proteins shown to be expressed and/or function in guard cells based on literature were identified in our guard cell proteome.** | | |
|---|---|---|
| **AGI** | **Name** | **Reference** |
| At1g11260 | STP1 | (Stadler et al., 2003) |
| At1g37130 | NIA2 | (Desikan et al., 2002) |
| At1g48270 | GCR1 | (Pandey and Assmann, 2004) |
| At2g18960 | OST2 | (Merlot et al., 2007) |
| At3g15730 | PLDα1 | (Mishra et al., 2006) |
| At3g45780 | PHOT2 | (Zhang et al., 2004) |
| At3g53720 | CHX20 | (Padmanaban et al., 2007) |
| At4g18290 | KAT2 | (Pilot et al., 2001) |
| At4g23650 | CPK3 or CDPK6 | (Mori et al., 2006) |
| At5g23060 | CAS | (Han et al., 2003) |
| At5g58140 | PHOT1 | (Kinoshita et al., 2001) |
| **B. Fifty-three proteins were predicted to be signaling proteins by GO software.** | | |
| AGI | Name | |
| At1g05810 | ARA-1, Ras-related protein/ small GTP-binding protein | |
| At1g06840 | leucine-rich repeat transmembrane protein kinase | |
| At1g09100 | 26S protease regulatory subunit 6A | |
| At1g52280 | Ras-related GTP-binding protein | |
| At1g71860 | PTP1, a protein with tyrosine phosphatase activity that is downregulated in response to cold and upregulated in response to salt stress | |
| At1g73670 | AtMPK15, member of MAP Kinase | |
| At1g75640 | leucine-rich repeat family protein | |
| At1g76030 | vacuolar ATP synthase subunit B | |
| At1g78580 | AtTPS1, an enzyme putatively involved in trehalose biosynthesis | |
| At2g04880 | ZAP1, member of WRKY Transcription Factor | |
| At2g16600 | ROC3, cytosolic cyclophilin | |
| At2g19860 | AtHXK2, hexokinase 2 | |
| At2g20610 | SUR1, confers auxin overproduction | |
| At2g21880 | Ras-related GTP-binding protein | |
| At2g25170 | PKL, a SWI/SWF nuclear-localized chromatin remodeling factor of the CHD3 group | |
| At2g26730 | leucine-rich repeat transmembrane protein kinase | |
| At2g31083 | CLE5, member of a large family of putative ligands homologous to the Clavata3 gene | |
| At2g32410 | similar to auxin-resistance protein AXR1 (AXR1) | |
| At2g36830 | GAMMA-TIP, encodes a tonoplast intrinsic protein | |
| At2g37970 | SOUL heme-binding family protein | |
| At2g44050 | COS1, acts in the jasmonic acid signaling pathway. | |
| At2g46070 | AtMPK12, member of MAP Kinase | |
| At3g02880 | leucine-rich repeat transmembrane protein kinase | |
| At3g08510 | AtPLC2, phosphoinositide specific phospholipase C | |
| At3g08680 | leucine-rich repeat transmembrane protein kinase | |

| | |
|---|---|
| At3g15060 | Ras-related GTP-binding family protein |
| **At3g15730** | PLDα1, phospholipase D alpha 1 |
| At3g18040 | AtMPK9, MAP kinase 9 |
| At3g18820 | Ras-related GTP-binding protein |
| At3g46060 | ARA3, Ras-related protein/ small GTP-binding protein |
| At3g53020 | STV1, ribosomal protein L24 |
| At3g55020 | RabGAP/TBC domain-containing protein |
| At3g62030 | ROC4, nuclear-encoded chloroplast stromal cyclophilin |
| At4g01370 | AtMPK4, MAP kinase involved in mediating responses to pathogens |
| At4g02080 | SAR1A, GTP-binding protein |
| At4g03550 | AtGSL5, a callose synthase that is required for wound and papillary callose formation in response to fungal pathogens Erysiphe and Blumeria |
| At4g15900 | PRL1, mutations confer hypersensitivity to glucose and sucrose and augments sensitivity to cytokinin |
| At4g18430 | Ras-related GTP-binding protein |
| **At4g23650** | CPK3 or CDPK6, member of Calcium Dependent Protein Kinase |
| At4g28650 | leucine-rich repeat transmembrane protein kinase |
| At4g29810 | AtMKK2, MAP kinase kinase 2 |
| At4g33680 | AGD2, aminotransferase class I and II family protein |
| At4g34870 | ROC5, belongs to cyclophilin family |
| At4g38690 | 1-phosphatidylinositol phosphodiesterase-related |
| At4g38740 | ROC1, cytosolic cyclophilin |
| At5g16590 | leucine-rich repeat transmembrane protein kinase |
| At5g19390 | similar to pleckstrin homology (PH) domain-containing protein / RhoGAP domain-containing protein |
| At5g39500 | pattern formation protein |
| At5g53320 | leucine-rich repeat transmembrane protein kinase |
| At5g58440 | phox (PX) domain-containing protein |
| At5g60600 | GCPE, encodes a chloroplast-localized hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP) synthase (HDS) |
| At5g63310 | NDPK2, maintains intracellular dNTP levels except ATP |
| At5g67030 | ABA1, zeaxanthin epoxidase (ZEP) |

Bolded proteins in part B represent proteins which are also present in part A.

**Table 2-6. Seventy-one guard cell specific proteins were identified.**

**A. Eight proteins were identified under certain conditions by MS technology.**

| AGI | Protein name | Reference |
|---|---|---|
| At1g50010 | TUA2 (tubulin alpha-2 chain) | (Sorin et al., 2006) |
| At3g16080 | 60S ribosomal protein L37 (RPL37C) | (Carroll et al., 2008) |
| At3g18040 | MPK9 | (Heazlewood et al., 2004) |
| At3g23840 | transferase family protein | (Friso et al., 2004) |
| At5g08670 | ATP synthase beta chain 1, mitochondrial | (Dixon et al., 2005) |
| At5g08690 | ATP synthase beta chain 2, mitochondrial | (Job et al., 2005) |
| At5g54190 | PORA (Protochlorophyllide reductase A) | (Philippar et al., 2007) |
| AtMg01190 | ATPase subunit 1 | (Rajjou et al., 2004) |
| **B. Twelve proteins have been shown to function in any other tissues by mutant analysis**. | | |
| At1g07780 | PAI1 | (Melquist and Bender, 2004) |
| At1g48270 | GCR1 | (Pandey and Assmann, 2004) |
| At1g62360 | STM | (Clark et al., 1996) |
| At1g65860 | A FMO family protein | (Hansen et al., 2007) |
| At2g29980 | FAD3 | (Browse et al., 1993) |
| At2g46800 | ATMTP1 | (Desbrosses-Fonrouge et al., 2005) |
| At3g52590 | UBQ1 | (Johnson et al., 2004) |
| At3g54560 | HTA11 | (March-Diaz et al., 2008) |
| At3g55830 | EPC1 | (Bown et al., 2007) |
| At5g04110 | DNA topoisomerase II family protein | (Wall et al., 2004) |
| At5g40780 | LHT1 | (Svennerstam et al., 2007) |
| At5g59820 | RHL41 | (Davletova et al., 2005) |
| **C. Fifty-one proteins have not been studied.** | | |
| At1g01500 | unknown protein | |
| At1g06030 | pfkB-type carbohydrate kinase family protein | |
| At1g06630 | F-box family protein | |
| At1g07350 | transformer serine/arginine-rich ribonucleoprotein, putative | |
| At1g08135 | CHX6B | |
| At1g10770 | invertase/pectin methylesterase inhibitor family protein | |
| At1g16140 | WAKL3, similar to wall-associated kinase protein family | |
| At1g21540 | AMP-binding protein, putative | |
| At1g32120 | unknown protein | |
| At1g50700 | CPK33 (calcium-dependent protein kinase 33) | |
| At1g51490 | glycosyl hydrolase family 1 protein | |
| At1g53290 | galactosyltransferase family protein | |
| At1g57620 | emp24/gp25L/p24 family protein | |
| At1g63310 | unknown protein | |
| At1g67580 | protein kinase family protein | |
| At1g67640 | lysine and histidine specific transporter, putative | |
| At1g73670 | ATMPK15 | |
| At2g02540 | ATHB21/ZFHD4 | |

| | |
|---|---|
| At2g07785 | NADH-ubiquinone oxidoreductase, putative |
| At2g31083 | CLE5 |
| At2g46320 | mitochondrial substrate carrier family protein |
| At3g05030 | NHX2 |
| At3g06410 | nucleic acid binding |
| At3g17070 | peroxidase, putative |
| At3g26430 | GDSL-motif lipase/hydrolase family protein |
| At3g26740 | CCL (CCR-LIKE) |
| At3g28530 | UDP-glucose 4-epimerase |
| At3g42170 | transposase-like gene |
| At3g42950 | glycoside hydrolase family 28 protein |
| At4g02420 | lectin protein kinase, putative |
| At4g05590 | unknown protein |
| At4g07830 | transposable element gene |
| At4g11660 | AT-HSFB2B |
| At4g16590 | ATCSLA01 |
| At4g17100 | serine protease protein -related |
| At4g23900 | NDK4 |
| At4g28650 | leucine-rich repeat transmembrane protein kinase, putative |
| At4g31130 | unknown protein |
| At4g32280 | IAA29 |
| At5g02020 | unknown protein |
| At5g15210 | ZFHD3 |
| At5g50230 | nucleotide binding |
| At5g53320 | leucine-rich repeat transmembrane protein kinase, putative |
| At5g55040 | DNA-binding bromodomain-containing protein |
| At5g58340 | DNA binding |
| At5g59350 | unknown protein |
| At5g60720 | unknown protein |
| At5g62020 | AT-HSFB2A |
| AtCg00860 | unknown protein |
| AtCg01000 | unknown protein |
| AtMg00480 | subunit 8 of the mitochondrial F(O) ATP synthase complex |

**Table 2-7. Eleven previously identified biomarker proteins were identified in our guard cell proteome.**

| AGI | Organ biomarker | Name |
|---|---|---|
| AT1G26910 | flower | 60S ribosomal protein L10 (RPL10B) |
| AT1G30120 | silique | PDH-E1 BETA (PYRUVATE DEHYDROGENASE E1 BETA) |
| AT1G73600 | silique | phosphoethanolamine N-methyltransferase 3, putative (NMT3) |
| AT1G75040 | flower | PR5 (PATHOGENESIS-RELATED GENE 5) |
| AT1G78860 | root | curculin-like (mannose-binding) lectin family protein |
| AT1G80660 | flower | AHA9 (Arabidopsis H(+)-ATPase 9) |
| AT4G08780 | root | peroxidase, putative |
| AT4G13840 | silique | transferase family protein |
| AT5G23020 | root | MAM-L (METHYLTHIOALKYMALATE SYNTHASE-LIKE |
| AT5G60660 | root | PIP2;4/PIP2F (plasma membrane intrinsic protein 2;4) |
| AT5G66400 | seed | RAB18 (RESPONSIVE TO ABA 18) |

**Table 2-8. Sixty-seven proteins have been demonstrated to function in Arabidopsis guard cells based on published literature.**

| Protein Name | AGI | Reference |
|---|---|---|
| ABA2 | At1g52340 | (Xie et al., 2006) |
| ABH1 | At2g13540 | (Hugouvieux et al., 2001) |
| ABI1 | At4g26080 | (Allen et al., 1999) |
| ABI2 | At5g57050 | (Allen et al., 1999) |
| AGB1 | At4g34460 | (Fan et al., 2008a) |
| AHK5 | At5g10720 | (Desikan et al., 2008) |
| AKT2 | At4g22200 | (Ivashikina et al., 2005) |
| ARR2 | At4g16110 | (Desikan et al., 2006) |
| AtCAS | At5g23060 | (Han et al., 2003) |
| AtCHX20 | At3g53720 | (Padmanaban et al., 2007) |
| AtERF7 | At3g20310 | (Song et al., 2005) |
| AtGPX3 | At2g43350 | (Miao et al., 2006) |
| AtMRP4 | At2g47800 | (Klein et al., 2004) |
| AtMRP5 | At1g04120 | (Klein et al., 2003) |
| AtNOS | At3g47450 | (Bright et al., 2006) |
| AtPDR3 | At2g29940 | (Galbiati et al., 2008) |
| AtRAC1 | At2g17800 | (Lemichez et al., 2001) |
| AtrbohD | At5g47910 | (Kwak et al., 2003) |
| AtrbohF | At1g64060 | (Kwak et al., 2003) |
| AtSTP1 | At1g11260 | (Stadler et al., 2003) |
| CBL1 | At4g17615 | (Cheong et al., 2007) |
| CBL9 | At5g47100 | (Cheong et al., 2007) |
| CHL1 | At1g12110 | (Guo et al., 2003) |
| CIPK23 | At1g30270 | (Cheong et al., 2007) |
| CHLH | At5g13630 | (Shen et al., 2006) |
| COI1 | At2g39940 | (Munemasa et al., 2007) |
| CPK3 | At4g23650 | (Mori et al., 2006) |
| CPK6 | At2g17290 | (Mori et al., 2006) |
| CPK11 | At1g35670 | (Zhu et al., 2007) |
| DET3 | At1g12840 | (Allen et al., 2000) |
| EIN2 | At5g03280 | (Desikan et al., 2006) |
| ERA1 | At5g40280 | (Pei et al., 1998) |
| ETR1 | At1g66360 | (Desikan et al., 2005) |
| FLS2 | At5g46330 | (Melotto et al., 2006) |
| GCA2 | Unknown | (Allen et al., 2001) |
| GCR1 | At1g48270 | (Pandey and Assmann, 2004) |
| GCR2 | At1g52920 | (Liu et al., 2007) |
| GORK | At5g37500 | (Hosy et al., 2003) |
| GPA1 | At2g26300 | (Wang et al., 2001) |
| GRP7 | At2g21660 | (Kim et al., 2008) |

| | | |
|---|---|---|
| HAB1 | At1g72770 | (Saez et al., 2006) |
| HIC1 | At2g46720 | (Gray et al., 2000) |
| HT1 | At1g62400 | (Hashimoto et al., 2006) |
| KAT1 | At5g46240 | (Kwak et al., 2001) |
| KAT2 | At4g18290 | (Lebaudy et al., 2008) |
| MEK1 | At4g26070 | (Xing et al., 2007) |
| MYB44 | At5g67300 | (Jung et al., 2008) |
| MYB60 | At1g08810 | (Cominelli et al., 2005) |
| MYB61 | At1g09540 | (Liang et al., 2005) |
| NIA1 | At1g77760 | (Desikan et al., 2002) |
| NIA2 | At1g37130 | (Desikan et al., 2002) |
| OST1 | At4g33950 | (Mustilli et al., 2002) |
| OST2 | At2g18960 | (Merlot et al., 2007) |
| PHOT1 | At5g58140 | (Kinoshita et al., 2001) |
| PHOT2 | At3g45780 | (Kinoshita et al., 2001) |
| PHS1-3 | At5g23720 | (Quettier et al., 2006) |
| PIP5K4 | At3g56960 | (Lee et al., 2007b) |
| PLDα1 | At3g15730 | (Mishra et al., 2006) |
| RCN1 | At1g13320 | (Kwak et al., 2002) |
| RPK1 | At1g69270 | (Osakabe et al., 2005) |
| ROP10 | At3g48040 | (Zheng et al., 2002) |
| ROP2 | At1g20090 | (Jeon et al., 2008) |
| SDIR1 | At3g55530 | (Zhang et al., 2007) |
| SLAC1 | At1g12480 | (Negi et al., 2008) |
| SYP61 | At1g28490 | (Zhu et al., 2002) |
| TPC1 | At4g03560 | (Peiter et al., 2005) |
| TPK1 | At5g55630 | (Gobert et al., 2007) |

**Table 2-9. Proteins encoded by fifteen guard cell specific transcripts identified by Leonhardt et al. (2004), were identified in guard cell proteome.**

| AGI | Name |
|---|---|
| At1g21400 | 2-oxoisovalerate dehydrogenase |
| At1g68530 | involved in wax biosynthesis |
| At1g78580 | an enzyme putatively involved in trehalose biosynthesis |
| At2g04280 | expressed protein |
| At2g04880 | member of WRKY Transcription Factor |
| At2g21060 | glycine-rich protein (AtGRP2b) mRNA |
| At2g21590 | similar to glucose-1-phosphate adenylyltransferase large subunit 2 (APL2) / ADP-glucose pyrophosphorylase |
| At2g22480 | phosphofructokinase family protein |
| At2g26250 | epidermis-specific |
| At2g47240 | similar to long-chain-fatty-acid--CoA ligase |
| At2g47980 | expressed protein |
| At4g14480 | protein kinase family protein |
| At4g24510 | involved in C28 to C30 fatty acid elongation |
| At4g36250 | aldehyde dehydrogenase family protein |
| At4g39330 | similar to mannitol dehydrogenase |

**Table 2-10 Primer information.**

| Name | Primer sequence |
|---|---|
| iPGM1-1 LP | ATGTTCTATCAATCTCCGGGG |
| iPGM1-1 RP | TTGATGCTTTTTGGATTCATTG |
| iPGM1-2 LP | TCGCATTAAAATTATCACTCTTGTG |
| iPGM1-2 RP | AGAACCATCCAAAACATCACG |
| iPGM2-1 LP | GAGGTAGGCAATGCTTGTGAG |
| iPGM2-1 RP | TTACCTGGTCAAACTTGCCAC |
| iPGM2-2 LP | ATTGTCTGCAAACCAGGACAC |
| iPGM2-2 RP | GGTCCAATTGTTGATGGTGAC |
| LBb1 | GCGTGGACCGCTTGCTGCAACT |
| RB1 | ATTAAACTCCAGAAACCCGCGGCTGAG |
| RT iPGM1 LP (p1) | ATGGCTACCTCCTCCGCTTGG |
| RT iPGM1 RP (p2) | CTACTCCACTACTTCAATCAGGGT |
| RT iPGM1 RP (p3) | GTGGATACAGTTGTACTGATCAGG |
| RT iPGM1 RP (p4) | GAACTCCACCGTCACTGAGAAGTC |
| RT iPGM2 LP (p5) | ATGGGTAGCTCCGGCGACGTTAAC |
| RT iPGM2 RP (p6) | TCACTTCTCGACGACTTC |
| RT iPGM2 RP (p7) | CGAACGCCATTGTGTGCCAGAT |

# Chapter 3

## ABA-regulated G protein signaling in Arabidopsis guard cells: a proteomic perspective

In this chapter, Figure 3-4 was obtained by Dr. Laetitia Perfus. All other data were obtained by Zhixin Zhao.

**Abstract**

Signaling cascades mediated by heterotrimeric G proteins are ubiquitous and important signal transduction mechanisms in both metazoans and plants. In the model plant Arabidopsis, the sole canonical G protein α subunit, GPA1, has been implicated in multiple signaling events, including guard cell movement regulated by the plant stress hormone abscisic acid (ABA). However, only a handful of proteins have been demonstrated to be involved in GPA1 signaling to date. Here, we compared the proteome composition of guard cells from wild type Col vs *gpa1-4* null mutants with and without ABA treatment using iTRAQ technology to identify guard cell proteins whose abundance was affected by ABA and/or GPA1. After imposition of strict selection criteria, the abundance of two proteins in Col and six proteins in *gpa1-4* was found to be affected by ABA in guard cells, and 18 guard cell proteins were quantitatively affected by the mutation of *GPA1*. Based on known functions of the differentially expressed proteins, our data suggest that GPA1 inhibits guard cell photosynthesis and promotes the availability of reactive oxygen species (ROS) in guard cells. To our knowledge, this is the first time that iTRAQ has been applied to quantitatively study protein abundance in a single cell type in plants.

## Introduction

Abscisic acid (ABA), one of the five major plant hormones, regulates many aspects of plant growth and development, including seed dormancy and germination, early seedling development and stomatal movements (Giraudat et al., 1994; Himmelbach et al., 1998; Xie et al., 2005). Under stress conditions, especially under drought stress, accumulation of ABA in plants facilitates acclimation responses of plants to environment changes (Seo and Koshiba, 2002). The global effect of ABA on the plant transcriptome

has been widely studied (Seki et al., 2002; Leonhardt et al., 2004; Li et al., 2006), however, no global analysis of the effect of ABA on the plant proteome has been reported to date. Here, for the first time, we studied global changes in protein abundance induced by ABA in a single cell type, guard cells, in both wild type Col and a mutant lacking the Gα subunit.

Heterotrimeric G proteins, composed of the three different subunits α, β and γ, are major components of signal transduction pathways in both mammalian and plant systems. In the canonical model in mammalian cells, Gα couples with guanosine diphosphate (GDP) and associates with the Gβγ dimer when inactive. When activated by G protein coupled receptors (GPCRs), Gα dissociates from the Gβγ dimer and GDP is replaced by GTP. Upon dissociation, either Gα or the Gβγ dimer can act as a functional unit and induce downstream signaling. In contrast to mammalian cells, where multiple α, β and γ genes exist, there is only one prototypical Gα (*GPA1*), one Gβ (*AGB1*) and two known Gγ (*AGG1* and *AGG2*) genes in Arabidopsis (Assmann, 2002, 2004). Despite the comparative simplicity of players, G proteins have been shown to participate in multiple signaling pathways in Arabidopsis, including many that are regulated by ABA, notably seed germination and early seedling development (Lapik and Kaufman, 2003; Pandey et al., 2006), guard cell signaling (Wang et al., 2001; Fan et al., 2008), and oxidative stress responses (Chen et al., 2004; Joo et al., 2005).

Mutants lacking the Gα subunit show phenotypes in multiple aspects of guard cell function, including hyposensitivity to ABA-inhibition of inward $K^+$ channels and stomatal opening (Wang et al., 2001; Fan et al., 2008), hyposensitivity to ABA-activation of anion channels through a pH-independent pathway (Wang et al., 2001), hyposensitivity to sphingosine-1-phosphate (S1P)-regulation of stomatal movements and ion channel activities (Coursol et al., 2003) and hyposensitivity to phyto-S1P-regulation of stomatal movements (Coursol et al., 2005). Since we are particularly interested in G-protein signaling in guard cells, in this study, we used a null mutant lacking the Gα subunit in the Col background, *gpa1-4*, to study proteins whose abundance is affected by loss of *GPA1* in Arabidopsis guard cells.

The strength of comparative proteomics lies in its ability to reveal quantitative protein changes between samples on a large scale. Isotope tags for relative and absolute quantification (iTRAQ) is a gel-free method for quantitative comparisons of protein samples. The iTRAQ reagent (Applied Biosystems) labels lysine residues and the N termini of peptides with specific reporter moieties and can be used for simultaneous comparison of multiple samples. Upon fragmentation of the labelled proteins by MS/MS, the relative quantity of each peptide is correlated to the relative intensity of the reporter peaks, and thus to the protein it was derived from in that sample (Schneider and Hall, 2005). To date only four papers have reported using iTRAQ to study protein abundance in Arabidopsis (Dunkley et al., 2006; Jones et al., 2006; Rudella et al., 2006; Rutschow et al., 2008).

The main objective of this study was to discover candidate proteins involved in ABA and/or GPA1 signaling in guard cells using iTRAQ technology. We first showed that iTRAQ is efficient in identifying Arabidopsis leaf proteins. Since it was shown previously that GPA1 is required for proper execution of ABA-signaling pathways in guard cells, we then compared protein abundances from wild type Col and *gpa1-4* guard cell protoplasts (GCPs) with and without 50 μM ABA treatment. ABA treatment significantly affected the amounts of two proteins in Col guard cells while affecting six proteins in *gpa1-4*. Eighteen proteins were significantly affected in abundance in *gpa1-4* guard cells compared to Col. ABA and GPA1 signaling models are proposed on the basis of our iTRAQ study. The reproducibility of the iTRAQ technology and the reported fold changes identified by the iTRAQ technology in plant iTRAQ studies are also discussed.

## Results

### Proteins identified in Arabidopsis Col leaves

Because iTRAQ technology has not been extensively applied to plant systems, we first evaluated its usefulness and reproducibility in identification of Arabidopsis leaf proteins. Two Col leaf samples were in-solution digested and labeled with two different iTRAQ reagents. The differentially labeled peptides from these two replicate samples were pooled together and analyzed using LC-MS/MS. In total, 249 proteins were identified with a false discovery rate (FDR) < 0.05 and confidence interval (C.I) = 99.99% by Protein Pilot software. Among the 249 proteins identified, only seven proteins showed $P < 0.05$, and these had relative ratios between 0.85-1.17, while 242 proteins (~97%) exhibited no significant changes in abundance (**Table 3-1**). These results suggest that the iTRAQ technology is highly reproducible in identification and quantification of Arabidopsis leaf proteins.

### Proteins identified in Arabidopsis guard cells

To globally view protein profile differences affected by ABA or *GPA1* null mutation in guard cells, iTRAQ experiments were performed using proteins from Col and *gpa1-4* guard cell protoplasts (GCPs) with or without ABA treatment. In total, 48 million GCPs from 4800 healthy Arabidopsis leaves were isolated (12 million GCPs for each sample). Guard cell protoplasts from each genotype with or without ABA treatments were isolated side-by-side by adding 50 μM (final concentration) ethanol (solvent control) or ABA in the enzyme solutions during the protoplast digestion processes for control or ABA-treated samples respectively. Total proteins were extracted from GCPs and for each sample, 100 μg of the proteins were trypsin digested and labeled with an iTRAQ reagent (see Material and methods for details). For the 1$^{st}$ and 2$^{nd}$ replicate, peptides derived from

Col guard cell proteins without and with ABA treatment were labeled with iTRAQ reagents 114 and 115 respectively, and peptides derived from *gpa1-4* guard cell proteins without and with ABA treatment were labeled with reagents 116 and 117 respectively. To reduce any potential variation introduced by the labeling reaction, iTRAQ labeling was swapped in the 3rd replicate, using reagents 114 and 115 for *gpa1-4* guard cell proteins without and with ABA treatment, and 116 and 117 for Col guard cell proteins without and with ABA treatment. Histograms of the log-transformed peptides ratios (**Figure 3-1**) revealed that no bias had been introduced via the iTRAQ labeling reaction.

In summary, three independent biological replicates were performed with four samples differentially labeled with iTRAQ reagents 114, 115, 116, 117. Using Protein Pilot software to search the NCBI Arabidopsis database, 351, 291 and 224 proteins were identified from the 1st, 2nd and 3rd biological replicates respectively with FDR < 0.05 and C.I = 99.99% (**Table 3-2**). In total, 454 proteins were identified in Arabidopsis guard cells, with 144 proteins identified in all three samples. To be considered as significantly expressed in two samples, we imposed the following stringent criteria to the identified proteins: 1) the iTRAQ ratios must be higher than 1.17 or lower than 0.85 (i.e. the changes in abundance are greater than the maxima observed in our replicate leaf samples), and 2) the P values of the iTRAQ ratios must be less than 0.05, and 3) proteins are identified by at least two biological replicates.

**Proteins differentially expressed in guard cells upon mutation of *GPA1***

Compared to Col, 18 proteins were changed significantly in abundance in *gpa1-4* guard cells: only one protein (A5g55660) was down-regulated while 17 were up-regulated by mutation of *GPA1* in guard cells (**Table 3-3**). The down-regulated protein, At5g55660, has been previously identified in the mitochondrial proteome (Heazlewood et al., 2004). Paradoxically, this protein does not contain sequence predicted to target it to mitochondria, but contains sequences predicted to target it to the nucleus by SubLoc and WoLFPSORT prediction software, and unfortunately its function is still unknown. All of

the 17 quantitatively up-regulated proteins have been identified in the Arabidopsis chloroplast proteome (Kleffmann et al., 2004), although three of these proteins are also predicted to localize elsewhere in the cell (**Table 3-3**). Of these 17 proteins, 12 are involved in photosynthesis: six are involved in the Calvin cycle, three are involved in the light reactions of photosynthesis, two are ATP synthases, and one is involved in electron transfer. Interestingly, the other five proteins increased in *gpa1-4* guard cells are all members of the Gene Onotology biological process category "response to stress". CAT3 (At1g20620) catalyzes the conversion of $H_2O_2$ into water and oxygen, PDIL1-4 (At5g60640) is involved in protein folding, ROC4 (At3g62030) functions in protein synthesis and folding, TGG2 (At5g25980) hydrolyzes glucosinolates and ERD14 (At1g76180) is a dehydrin protein. While ERD14 abundance was upregulated in *gpa1-4* guard cells as compared to Col guard cells, its abundance was downregulated by ABA treatment in *gpa1-4* (but not Col) guard cells upon ABA treatment. None of the other 17 proteins was quantitatively affected by ABA treatment, regardless of plant genotype (see next section).

**Proteins with differential expression relative to ABA treatment**

Two proteins in Col guard cells exhibited differential abundances in response to ABA (**Table 3-4a**). Malate dehydrogenase (At1g53240, MDH), which catalyzes the interconversion of oxaloacetate and malate, is one of the two proteins with increased abunance in Col in response to ABA. MDH reduces oxaloacetate to malate in mitochondria while oxidizing malate to oxaloacetate in the cytosol, and it is involved in the TCA cycle and gluconeogenesis. The second protein overrepresented in ABA-treated Col is RubisCO small subunit 2B (At5g38420, rbcS2B). While the specific function of rbcS2B in Arabidopsis is unclear, as a subunit of RuBisCO, it catalyzes the fixation of $CO_2$ with D-ribulose-1,5-bisphosphate (RuBP) in the Calvin Cycle, and catalyzes combination of $O_2$ with RuBP in photorespiration process (Spreitzer and Salvucci, 2002).

While the protein amounts of MDH and rbcS2B were increased by ABA treatment in Col guard cells, but not affected by ABA in *gpa1-4* guard cells.

Six proteins in *gpa1-4* guard cells exhibited differential abundances in response to ABA (**Table 3-4a**). These four upregulated and two downregulated proteins in protein amount by ABA in *gpa1-4* guard cells were all identified in the chloroplast proteome by Kleffmann et al., (2004). Five of the six proteins participate in the light reactions of photosynthesis (PsaA, PsaB, PsbB, PsbC, Lhcb6) and the remaining protein is ERD14, a dehydrin protein. PsaA, PsaB, PsbB and PsbC are chloroplast encoded proteins, and photosystem subunits while Lhcb6 is an antennae protein in photosystem II (**Table 3-4b**). None of these five proteins was quantitatively affected by ABA in Col guard cells.

## Discussion

### Reproducibility of iTRAQ technology in identification of Arabidopsis proteins

To evaluate the reproducibility and usefulness of the iTRAQ method in studying protein abundance changes in Arabidopsis, two replicate Col leaf samples were pooled together after differential iTRAQ labeling and analyzed in the same LC-MS/MS process. iTRAQ results showed that 242 out of 249 proteins did not differ significantly ($P < 0.05$) in abundance between these two samples, strongly indicating the high reproducibility of iTRAQ technology in studying protein changes in Arabidopsis. Compared to previously identified leaf proteomes, 238 out of the 249 proteins were previously identified by Lee et al., (Lee et al., 2007) and 239 out of the 249 proteins were previously identified as present in the leaf proteome by Baerenfaller et al. (Baerenfaller et al., 2008). The leaf proteins newly identified by our study are given in **Table 3-1**. These results indicate that iTRAQ is a good approach in identification of Arabidopsis leaf proteins and can quantify protein amount changes in Arabidopsis with high reproducibility.

We are interested in Arabidopsis guard cell proteins whose abundance is regulated by ABA with and without *GPA1* mutation, and proteins whose levels change significantly in response to *GPA1* mutation. Therefore, four protein samples from Col and *gpa1-4* GCPs with and without ABA treatment were prepared with iTRAQ labeling. Three independent experiments were performed for each treatment/genotype combination. Comparison of histograms of the log-transformed peptides ratios suggested that the iTRAQ labeling did not introduce noise for comparison of protein abundance in all three replicates (**Figure 3-1**).

In total, 351, 291 and 224 proteins were identified from the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ experiment respectively, and 220 proteins were identified simultaneously from the 1$^{st}$ and 2$^{nd}$ experiment, 172 from the 1$^{st}$ and 3$^{rd}$ experiments, 159 from the 2$^{nd}$ and 3$^{rd}$ experiment and 144 proteins from all three experiments (**Figure 3-2**). These results showed that the overlap between any two replicates is 49 to 71%.

Independent of the iTRAQ labeling process, the reproducible identification of proteins in replicate iTRAQ experiments is also strongly affected by the shotgun method of proteome identification. The reproducibility and comprehensiveness of the shotgun method has been studied by several groups (Berg et al., 2006; Chen et al., 2007). One good example is the study of the human liver proteome (Chen et al., 2007) in which six replicates of LC MS/MS were performed to obtain a comprehensive human liver proteome. Comparison of proteins identified from any two replicate runs showed only 25-40% overlap between any two replicates. Berg et al. (2006) also showed ~30-70% overlap between any two replicates in their study of membrane proteins extracted from Arabidopsis callus cultures. While these studies by Chen et al. and Berg et al. utilized only technical replicates for their shotgun analyses, we used biological replicates for our iTRAQ study, which have the potential for even higher levels of variation. This comparison strongly suggests that the reproducibility of the iTRAQ technology is satisfactory in our study, and any data variability is predominantly affected by the non-comprehensive nature of the shotgun method rather than by the iTRAQ labeling reaction. This conclusion is also supported by our LC-MALDI MudPIT data obtained by studying the WS guard cell proteome (Chapter 2), in which the overlap between the two

independent biological replicates was 50-60%. Biological variation can also affect reproducibility. To minimize the biological variation inherent in any two independent biological experiments, all steps were carefully controlled: all Arabidopsis plants were grown in the same growth chamber, leaves were cut at the same time every day, guard cell protoplasts were always isolated using identital enzyme digestion solutions and digestion times, and extraction of total proteins and peptide labeling were performed in parallel for all samples.

**Fold changes of protein abundance in iTRAQ studies**

Since the seven proteins out of 249 proteins (2.8%) with $P < 0.05$ in our replicate leaf vs. leaf comparison had iTRAQ fold change ratios ranging from 0.85 to 1.17, we decided to impose the cut-off criteria of a fold-change > 1.17 or < 0.85 and simultaneous identification by at least two iTRAQ experiments as our cut-off criteria, in addition to requiring a C.I of 99.99%. These criteria are similar to those imposed by Rutschow et al. (2008) in their study of a chloroplast protein mutant, and are more stringent than all other plant iTRAQ studies that we identified from the current literature (**Table 3-5**).

In our guard cell iTRAQ study, the relative protein abundance ratios for comparisons with $P < 0.05$ varied from 0.6-2.8. To date, only two studies have been reported to globally quantify protein changes in Arabidopsis (Jones et al., 2006; Rutschow et al., 2008). In one study, fold changes in protein abundances between control and three bacterial challenges ranged from 0.5 to 3 ($P < 0.05$) (Jones et al., 2006). In the study of Rutschow et al., (2008), fold-changes of all proteins identified in two replicates ranged from 0.59-2.07. These results suggest that the range in iTRAQ ratios found in our study compares well to ranges reported in other studies using the iTRAQ method. Two other relevant applications of iTRAQ in plants are also discussed here although P values for the relative protein ratios were not provided for those studies. Chloroplast membrane proteins were quantitatively compared between maize mesophyll and bundle sheath cells, and 101 out of the identified 116 proteins had abundance ratios within the range of 0.5-2 (Majeran

et al., 2008). The second study compared the abundance of proteins from roots of boron-tolerant and -intolerant barley plants, and found that only 11 peptides (derived from seven proteins) out of the 1038 identified peptides (~1%) had more than 2.5 fold changes (Patterson et al., 2007). These results suggest that, even without considering P values, most of the proteins identified via the iTRAQ method exhibit less than two fold changes. If, like the majority of the studies reported in **Table 3-5**, we remove the P value constraint in our study, we can identify 209 proteins with relative abundance ratios between 0.18-3.3 when *GPA1* is mutated in guard cells (**Table 3-2**). Together with these previous studies, our results suggest that less than two fold changes in protein abundance in identification of plant proteins are common in iTRAQ studies, and consideration of P values for comparison of protein abundance is advisable for iTRAQ studies. Consistent with this conclusion, Majeran et al. showed that fold changes in protein abundance determined by the iTRAQ method were relatively smaller than those determined by blue native gel and label-free methods (Majeran et al., 2008), suggesting that the iTRAQ method under-reports fold changes.

### GPA1 signaling in Arabidopsis guard cells

As shown in **Table 3-3**, 18 guard cell proteins were significantly affected by loss of *GPA1*. Of these, 17 out of the 18 proteins (94%) were also identified as members of the Arabidopsis chloroplast proteome (Kleffmann et al., 2004), although not all of these proteins reside exclusively in the chloroplast (**Table 3-3**). All 17 proteins were quantitatively increased in *gpa1-4* guard cells compared to Col guard cells. When the guard cell proteome identified in this study (454 proteins) was compared to the previously identified chloroplast proteome (Kleffmann et al., 2004), 29.5% (134 proteins) of the identified guard cell proteins (454 proteins) were also identified from the Arabidopsis chloroplast, yet only 17 (12.7%) out of the 134 identified chloroplast proteins were significantly up-regulated by mutation of *GPA1* (**Table 3-3**). This comparison demonstrates that the up-regulation of these 17 chloroplast proteins was

induced by the mutation of *GPA1* and not by differential contamination by the chloroplast-rich mesophyll cells in Col vs. *gpa1* samples, as any differential mesophyll cell contamination would cause a similar skew to all the identified chloroplast proteins. Importantly, no difference in carbon assimilation was detected between *gpa1-4* and Col leaves (Nilson and Assmann, unpub data), suggesting that photosynthetic rate is the same in Col and *gpa1-4* mesophyll cells. Of these 17 guard cell proteins, 12 are involved in photosynthesis, suggesting that photosynthetic activity may be repressed by GPA1 specifically in guard cells.

Guard cells have specialized chloroplasts, with high capacity for photophosphorylation and low capacity for carbon fixation (Shimazaki et al., 2007). Photophosphorylation by guard cell chloroplasts provide ATP and NADPH for the high energetic requirements of stomatal opening under light (Shimazaki et al., 2007). The observation that three proteins (PsbP1, PsbR, PSBO1) involved in the light reactions of photosynthesis and two chloroplastic ATP synthase proteins are of lower abundance in wild-type guard cells than in *gpa1* null mutant guard cells suggests that the presence of GPA1 may indirectly suppress photophosphorylation in guard cells, leading to the prediction that light-stimulated stomatal opening may be enhanced in *gpa1* null mutants.

The presence of GPA1 in wild-type guard cells also led to lower levels of five Calvin cycle proteins (**Table 3-3**) as compared to *gpa1* mutant guard cells. Although carbon fixation is generally accepted to operate at a greatly reduced level in guard cell chloroplasts as opposed to mesophyll cell chloroplasts (Shimazaki et al., 2007), the Calvin cycle may still contribute in a limited manner to production of osmotically-active carbohydrates (e.g. sucrose, malate) that would drive guard cell swelling and stomatal opening. Thus, these data also lead to the prediction that light-stimulated stomatal opening may be enhanced in *gpa1* mutants.

Finally, it was recently shown that GPA1 interacts with the plastid membrane protein THF1, and it has been hypothesized that this interaction functions in glucose sensing pathways in Arabidopsis seedlings (Huang et al., 2006). Therefore, one can speculate that a mechanism whereby GPA1 feeds back to suppress the abundance of certain photosynthetic proteins and thus (potentially) photosynthetic rates may be

regulated by glucose levels. In addition, glucose derived from starch breakdown serves as the precursor of malate, which is a major osmoticum produced during light-stimulated stomatal opening (Shimazaki et al., 2007). Therefore, GPA1 might be involved in the regulation of malate production by way of affecting glucose sensing in Arabidopsis guard cells (**Figure 3-3**).

As an initial, admittedly indirect, test of these scenarios, we evaluated light-stimulated stomatal opening in the presence of high vs. low concentrations of KCl (**Figure 3-4**). Under high KCl concentrations, energetic requirements for stomatal opening are lessened (Humble and Hsiao, 1970; Outlaw, 1983) and $Cl^-$ more readily substitutes for $malate^{2-}$ as the counterion balancing $K^+$ uptake (Travis and Mansfield, 1979). Under these high KCl conditions no difference between wild-type and *gpa1* null mutants was seen in the extent of light-stimulated stomatal opening. However, under low KCl conditions, which impose greater requirements for energy and malate production to drive stomatal opening, stomatal apertures of *gpa1* mutants were greater than wild-type. These results are consistent with the above hypotheses. We fully acknowledge, however, that these experiments are not definitive of mechanism, and that future detailed studies of guard cell photosynthetic reactions (Goh et al., 1999) in wild-type vs. *gpa1* mutant guard cells are needed.

The five non-photosynthetic proteins that showed elevated levels in *gpa1* guard cells are CAT3, TGG2, PDIL1-4, ROC4, and ERD14, while an unknown mitochondrial protein, At5g55660, is suppressed in *gpa1* guard cells. There are three catalases in Arabidopsis, CAT1, CAT2 and CAT3. Catalases act as $H_2O_2$ scavengers, converting $H_2O_2$ into water and oxygen (Frugoli et al., 1996). ROC4 (also named CYP20-3) is a member of the cyclophilin family of proteins. ROC4 has reductant activity in the chloroplast and can regenerate reduced peroxiredoxins (Prx) - the active form of peroxiredoxins (Laxa et al., 2007). Peroxiredoxins, similar to CAT3, are enzymes that decompose $H_2O_2$ (Dietz et al., 2006). PDIL1-4 is a disulphide isomerase-like protein. Protein disulphide isomerases (PDIs) assist in the formation of correct disulfide bonds during protein folding. ROC4 also posess peptidyl-prolyl *cis–trans* isomerase (PPI) activity. Correct formation of disulfide bonds and cis-trans isomerization of the peptide

bonds preceding proline are important and slow steps during protein folding process and these two steps are accelerated by PDI and cyclophilins respectively. PDI activity can be increased in the presence of enzymatic activity of PPI, indicating some synergism between the two (Schonbrunner and Schmid, 1992). It might be possible that PDIL1-4 together with ROC4 affects the active form of Prx and then further regulates the intracellular ROS concentration. ERD14 is a member of the dehydrin class of proteins, which are known to be upregulated in response to stress and are thought to play a protective role (Battaglia et al., 2008). Some dehydrin proteins have been shown to act as antioxidative proteins, scavenging hydroxyl and peroxyl radicals (Hara et al., 2004). The elevation of these five proteins in *gpa1* guard cells suggests that the redox status of *gpa1* guard cells may differ from that of wild-type guard cells. Indeed, there is already evidence in the literature supporting this hypothesis: *gpa1* guard cells have a reduced ability to produce ROS in response to ozone (Joo et al., 2005) and extracellular calmodulin (Chen et al., 2004).

Some dehydrins, including ERD14, are metal-binding proteins (Battaglia et al., 2008). The metal-binding activities of dehydrins may not only modulate redox-based signaling mechanisms (Hara et al., 2004). but also modulate other signaling cascades: ERD14 has been demonstrated to be a $Ca^{2+}$ binding protein and might act as an intracellular $Ca^{2+}$ buffer (Alsheikh et al., 2003). In guard cells, cytosolic $Ca^{2+}$ elevation can occur in response to ABA. $Ca^{2+}$ is an important signaling element in guard cells, and is known to inhibit stomatal opening and maintain stomatal closure by inhibition of $K^+$ influx channels and activation of anion efflux channels (Israelsson et al., 2006). Likewise, inhibition of $K^+$ influx channels and activation of anion efflux channels is initiated by ABA treatment in wild-type guard cells; however such ABA-mediated ion channel regulation is missing from *gpa1* mutant guard cells (Wang et al., 2001; Fan et al., 2008).

The last protein that is upregulated in *gpa1* guard cells is TGG2. TGG2 is one of the two functional myrosinase proteins (TGG1 and TGG2) in Arabidopsis leaves. TGG1 (but not TGG2) was detected as a highly abundant protein in guard cells (Chapter 2) and showed decreased protein abundance with mutation of *GPA1* in the 2$^{nd}$ replicate (*gpa1-4*/Col =0.8), however, iTRAQ ratios were not available for the 1$^{st}$ and 3$^{rd}$ replicates for

unknown reasons. These results suggest that the final effect of *GPA1* mutation on myrosinase concentration in guard cells, and the relationship between TGG2 function and mutation of *GPA1* require further investigation.

**ABA signaling in Arabidopsis guard cells**

Four of proteins (PLDα1, OST2, CHX20 and TGG1) previously shown to be involved in ABA-regulation of stomatal movements via mutant analysis (**Table 3-6**) were identified as present in the guard cell proteome in our iTRAQ study. However, none of these four proteins was significantly quantitatively affected by ABA in either Col or *gpa1* guard cells. Interestingly, previous reports showed that ABA stimulates PLDα1 activity (Jacob et al., 1999; Zhang et al., 2004), inhibits the activity of the *OST2* encoded $H^+$-ATPase (Merlot et al., 2007), and affects TGG1 function by either repartitioning of glucosinolates or by enhancement of myrosinase activity or substrate affinity (Chapter 2). These results, combined with ours, suggest that ABA likely regulates these proteins post-translationally, as opposed to regulating their abundance. Consistent with a scenario in which ABA regulation of stomatal movements does not depend strongly on regulation of protein abundance, only limited numbers of proteins (two in Col and six in *gpa1-4* guard cells) were identified to be significantly affected by ABA in protein abundance, although we note that it also remains possible that ABA-regulated proteins of overall low abundance were not detected in our shotgun sequencing.

Protein abundances of MDH and rbcS2B were increased by ABA in Col guard cells. ABA-related functions of these proteins have yet to be described. Since the protein amounts of MDH and rbcS2B were not affected by ABA in *gpa1-4* guard cells and it was shown previously that GPA1 acts downstream of ABA in guard cells (Wang et al., 2001; Fan et al., 2008), it is proposed that the presence of GPA1 is required for ABA-induced increases in these proteins. Since MDH catalyzes the interconversion of malate and oxaloacetate, it is possible that regulation of MDH protein abundance contributes to regulation of malate levels in guard cells.

Of the six proteins showing ABA-induced changes in *gpa1-4* guard cells, four were up-regulated by ABA. All four of these proteins are involved in the light reactions of photosynthesis, suggesting that photophosphorylation may be up-regulated by ABA in guard cells when *GPA1* is mutated. This could be a contributing factor to the observed hyposensitivity of *gpa1* guard cells to ABA-inhibition of stomatal opening in the light (Wang et al., 2001; Coursol et al., 2003; Fan et al., 2008). The only non-photosynthetic protein is ERD14, which was reduced in abundance in *gpa1-4* guard cells by ABA treatment, but was increased by comparison of *gpa1-4* guard cells to Col guard cells when both of which were treated with ABA.

In summary, we used the iTRAQ method in this study to discover proteins whose abundances are affected by ABA treatment and/or loss of *GPA1* in Arabidopsis guard cells. We demonstrated that the iTRAQ technique provides an efficient method for identification of new candidate proteins and models for guard cell signaling pathways in Arabidopsis. Our data suggest the hypotheses that ABA regulates stomatal movements mainly via post-translational mechanisms and/or that proteins which are quantitatively regulated by ABA are predominantly low abundance proteins and therefore not detected by our shotgun method.

## Material and methods

### Guard cell protoplast preparation and protein extraction

Plant growth conditions and GCP isolation procedures were the same as described by Zhao et al., (submitted). For control or ABA treated GCPs, 50 μM (final concentration) ABA or equivalent volume of ethanol was added during the enzyme digestion process in GCP preparation. 4 million GCPs from 400 leaves were used per genotype per treatment for protein extraction in each replicate. In total, 48 million GCPs from 4,800 leaves were used and proteins were extracted from GCPs according to Zhao et al. (submitted).

**Trypsin digestion and iTRAQ labeling**

One hundred μg of proteins from each sample were dissolved, reduced, alkylated and trypsin digested according to the protocol provided with the iTRAQ kit (Applied Biosystems, part #: 4374321). Peptides in each tube were labeled by one iTRAQ reagent as follow: for the 1st and 2nd replicates, peptides from Col GCPs without ABA were labeled by reagent 114; peptides from Col GCPs with 50 μM ABA treatment were labeled by reagent 115; peptides from *gpa1-4* GCPs without ABA were labeled by reagent 116 and peptides from *gpa1-4* GCPs with 50 μM ABA treatment were labeled with reagent 117; for the 3rd replicate, peptides from *gpa1-4* GCPs without ABA were labeled by reagent 114 and peptides from *gpa1-4* GCPs with 50 μM ABA treatment were labeled with reagent 115; peptides from Col GCPs without ABA were labeled by reagent 116; peptides from Col GCPs with 50 μM ABA treatment were labeled by reagent 117. All labeling steps were performed according to the protocol provided by Applied Biosystems. Before peptides from the four samples were pooled together, the labeling reaction was quenched by adding 100 μL distilled water, and then incubated at room temperature for 30 min. Three biological replicates were performed and compared.

**MS identification and data analysis**

All peptides were separated using LC-MALDI techniques through two sequential columns: strong cation exchange (SCX) and C18 nanoflow chromatography, and then identified by a 4800 proteomic analyzer. Peptide quantitation analyses were performed with Protein Pilot software (Applied Biosystems) using the following parameters: one missed cut of trypsin cleavage is allowed, all posttranslational modifications are allowed. All peptide spectra were searched against the NCBI Arabidopsis database. Local FDR for each protein was calculated (Tang et al., 2008). FDR and C.I were used to define the acceptable level of all the identified peptides. All peptides with FDR < 0.05 and C.I = 99.99% were used for further relative quantification assays. The relative quantification of

proteins is the ratio of peak areas at 114, 115, 116, 117, and proteins were only accepted that had relative quantification P values less than 0.05. The P value is a measurement of the certainty that the average ratios of a protein from two samples differ from one another. Identified proteins with quantification P values < 0.05, iTRAQ ratio of > 1.17 or < 0.85, and identification in at least two biological replicates were considered to have significant differences in protein abundance.

## References

**Alsheikh, M.K., Heyen, B.J., and Randall, S.K.** (2003). Ion binding properties of the dehydrin ERD14 are dependent upon phosphorylation. J Biol Chem **278,** 40882-40889.

**Assmann, S.M.** (2002). Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell **14 Suppl,** S355-373.

**Assmann, S.M.** (2004). Plant G proteins, phytohormones, and plasticity: three questions and a speculation. Sci STKE **2004,** re20.

**Baerenfaller, K., Grossmann, J., Grobei, M.A., Hull, R., Hirsch-Hoffmann, M., Yalovsky, S., Zimmermann, P., Grossniklaus, U., Gruissem, W., and Baginsky, S.** (2008). Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science **320,** 938-941.

**Battaglia, M., Olvera-Carrillo, Y., Garciarrubio, A., Campos, F., and Covarrubias, A.A.** (2008). The enigmatic LEA proteins and other hydrophilins. Plant Physiol **148,** 6-24.

**Berg, M., Parbel, A., Pettersen, H., Fenyo, D., and Bjorkesten, L.** (2006). Reproducibility of LC-MS-based protein identification. J Exp Bot **57,** 1509-1514.

**Chen, M., Ying, W., Song, Y., Liu, X., Yang, B., Wu, S., Jiang, Y., Cai, Y., He, F., and Qian, X.** (2007). Analysis of human liver proteome using replicate shotgun strategy. Proteomics **7,** 2479-2488.

**Chen, Y.L., Huang, R., Xiao, Y.M., Lu, P., Chen, J., and Wang, X.C.** (2004). Extracellular calmodulin-induced stomatal closure is mediated by heterotrimeric G protein and $H_2O_2$. Plant Physiol **136,** 4096-4103.

**Coursol, S., Fan, L.M., Le Stunff, H., Spiegel, S., Gilroy, S., and Assmann, S.M.** (2003). Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. Nature **423,** 651-654.

**Coursol, S., Le Stunff, H., Lynch, D.V., Gilroy, S., Assmann, S.M., and Spiegel, S.** (2005). Arabidopsis sphingosine kinase and the effects of phytosphingosine-1-phosphate on stomatal aperture. Plant Physiol **137,** 724-737.

**Dietz, K.J., Jacob, S., Oelze, M.L., Laxa, M., Tognetti, V., de Miranda, S.M., Baier, M., and Finkemeier, I.** (2006). The function of peroxiredoxins in plant organelle redox metabolism. J Exp Bot **57,** 1697-1709.

**Dunkley, T.P., Hester, S., Shadforth, I.P., Runions, J., Weimar, T., Hanton, S.L., Griffin, J.L., Bessant, C., Brandizzi, F., Hawes, C., Watson, R.B., Dupree, P., and Lilley, K.S.** (2006). Mapping the Arabidopsis organelle proteome. Proc Natl Acad Sci U S A **103,** 6518-6523.

**Fan, L.M., Zhang, W., Chen, J.G., Taylor, J.P., Jones, A.M., and Assmann, S.M.** (2008). Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ- and RGS-deficient Arabidopsis lines. Proc Natl Acad Sci U S A **105,** 8476-8481.

**Frugoli, J.A., Zhong, H.H., Nuccio, M.L., McCourt, P., McPeek, M.A., Thomas, T.L., and McClung, C.R.** (1996). Catalase is encoded by a multigene family in *Arabidopsis thaliana* (L.) Heynh. Plant Physiol **112,** 327-336.

**Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.C., Bouvier-Durand, M., and Vartanian, N.** (1994). Current advances in abscisic acid action and signalling. Plant Mol Biol **26,** 1557-1577.

**Goh, C.H., Schreiber, U., and Hedrich, R.** (1999). New approach of monitoring changes in chlorophyll *a* fluorescence of single guard cells and protoplasts in response to physiological stimuli. Plant, Cell & Environ **22,** 1057-1070.

**Hara, M., Fujinaga, M., and Kuboi, T.** (2004). Radical scavenging activity and oxidative modification of citrus dehydrin. Plant Physiol Biochem **42,** 657-662.

**Heazlewood, J.L., Tonti-Filippini, J.S., Gout, A.M., Day, D.A., Whelan, J., and Millar, A.H.** (2004). Experimental analysis of the Arabidopsis mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. Plant Cell **16,** 241-256.

**Himmelbach, A., Iten, M., and Grill, E.** (1998). Signalling of abscisic acid to regulate plant growth. Philos Trans R Soc Lond B Biol Sci **353,** 1439-1444.

**Huang, J., Taylor, J.P., Chen, J.G., Uhrig, J.F., Schnell, D.J., Nakagawa, T., Korth, K.L., and Jones, A.M.** (2006). The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in Arabidopsis. Plant Cell **18,** 1226-1238.

**Humble, G.D., and Hsiao, T.C.** (1970). Light-dependent influx and efflux of potassium of guard cells during stomatal opening and closing. Plant Physiol **46,** 483-487.

**Israelsson, M., Siegel, R.S., Young, J., Hashimoto, M., Iba, K., and Schroeder, J.I.** (2006). Guard cell ABA and $CO_2$ signaling network updates and $Ca^{2+}$ sensor priming hypothesis. Curr Opin Plant Biol **9,** 654-663.

**Jacob, T., Ritchie, S., Assmann, S.M., and Gilroy, S.** (1999). Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. Proc Natl Acad Sci U S A **96,** 12192-12197.

**Jones, A.M., Bennett, M.H., Mansfield, J.W., and Grant, M.** (2006). Analysis of the defence phosphoproteome of *Arabidopsis thaliana* using differential mass tagging. Proteomics **6,** 4155-4165.

**Joo, J.H., Wang, S., Chen, J.G., Jones, A.M., and Fedoroff, N.V.** (2005). Different signaling and cell death roles of heterotrimeric G protein α and β subunits in the Arabidopsis oxidative stress response to ozone. Plant Cell **17,** 957-970.

**Kaffarnik, F.A., Jones, A.M., Rathjen, J.P., and Peck, S.C.** (2008). Effector proteins of the bacterial pathogen *Pseudomonas syringae* alter the extracellular proteome of the host plant, *Arabidopsis thaliana*. Mol Cell Proteomics.

**Kleffmann, T., Russenberger, D., von Zychlinski, A., Christopher, W., Sjolander, K., Gruissem, W., and Baginsky, S.** (2004). The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr Biol **14,** 354-362.

**Kovacs, D., Kalmar, E., Torok, Z., and Tompa, P.** (2008). Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins. Plant Physiol **147,** 381-390.

**Lapik, Y.R., and Kaufman, L.S.** (2003). The Arabidopsis cupin domain protein AtPirin1 interacts with the G protein alpha-subunit GPA1 and regulates seed germination and early seedling development. Plant Cell **15,** 1578-1590.

**Laxa, M., Konig, J., Dietz, K.J., and Kandlbinder, A.** (2007). Role of the cysteine residues in *Arabidopsis thaliana* cyclophilin CYP20-3 in peptidyl-prolyl cis-trans isomerase and redox-related functions. Biochem J **401,** 287-297.

**Lee, J., Garrett, W.M., and Cooper, B.** (2007). Shotgun proteomic analysis of *Arabidopsis thaliana* leaves. J Sep Sci **30,** 2225-2230.

**Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G., and Schroeder, J.I.** (2004). Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell **16,** 596-615.

**Li, Y., Lee, K.K., Walsh, S., Smith, C., Hadingham, S., Sorefan, K., Cawley, G., and Bevan, M.W.** (2006). Establishing glucose- and ABA-regulated transcription networks in Arabidopsis by microarray analysis and promoter classification using a Relevance Vector Machine. Genome Res **16,** 414-427.

**Majeran, W., Zybailov, B., Ytterberg, A.J., Dunsmore, J., Sun, Q., and van Wijk, K.J.** (2008). Consequences of C4 differentiation for chloroplast membrane proteomes in maize mesophyll and bundle sheath cells. Mol Cell Proteomics. 2008 **7**, 1609-1638.

**Merlot, S., Leonhardt, N., Fenzi, F., Valon, C., Costa, M., Piette, L., Vavasseur, A., Genty, B., Boivin, K., Muller, A., Giraudat, J., and Leung, J.** (2007). Constitutive activation of a plasma membrane $H^+$-ATPase prevents abscisic acid-mediated stomatal closure. Embo J **26,** 3216-3226.

**Mishra, G., Zhang, W., Deng, F., Zhao, J., and Wang, X.** (2006). A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science **312,** 264-266.

**Outlaw, W.H.J.** (1983). Current concepts on the role of potassium in stomatal movements. Physiol. Planta **59,** 302-311.

**Padmanaban, S., Chanroj, S., Kwak, J.M., Li, X., Ward, J.M., and Sze, H.** (2007). Participation of endomembrane cation/$H^+$ exchanger AtCHX20 in osmoregulation of guard cells. Plant Physiol **144,** 82-93.

**Pandey, S., Chen, J.G., Jones, A.M., and Assmann, S.M.** (2006). G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. Plant Physiol **141,** 243-256.

**Patterson, J., Ford, K., Cassin, A., Natera, S., and Bacic, A.** (2007). Increased abundance of proteins involved in phytosiderophore production in boron-tolerant barley. Plant Physiol **144,** 1612-1631.

**Rudella, A., Friso, G., Alonso, J.M., Ecker, J.R., and van Wijk, K.J.** (2006). Downregulation of ClpR2 leads to reduced accumulation of the ClpPRS protease complex and defects in chloroplast biogenesis in Arabidopsis. Plant Cell **18,** 1704-1721.

**Rutschow, H., Ytterberg, A.J., Friso, G., Nilsson, R., and van Wijk, K.J.** (2008). Quantitative proteomics of a chloroplast SRP54 sorting mutant and its genetic interactions with CLPC1 in *Arabidopsis thaliana*. Plant Physiol.

**Schneider, L.V., and Hall, M.P.** (2005). Stable isotope methods for high-precision proteomics. Drug Discov Today **10,** 353-363.

**Schonbrunner, E.R., and Schmid, F.X.** (1992). Peptidyl-prolyl cis-trans isomerase improves the efficiency of protein disulfide isomerase as a catalyst of protein folding. Proc Natl Acad Sci U S A **89,** 4510-4513.

**Schwartz, A., and Zeiger, E.** (1984). Metabolic energy for stomatal opening. Roles of photophosphorylation and oxidative phosphorylation. Planta **161,** 129-136.

**Seki, M., Ishida, J., Narusaka, M., Fujita, M., Nanjo, T., Umezawa, T., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K.** (2002). Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. Functional & Integrative Genomics **2,** 282-291.

**Seo, M., and Koshiba, T.** (2002). Complex regulation of ABA biosynthesis in plants. Trends Plant Sci **7,** 41-48.

**Shimazaki, K., Doi, M., Assmann, S.M., and Kinoshita, T.** (2007). Light regulation of stomatal movement. Annu Rev Plant Biol **58,** 219-247.

**Spreitzer, R.J., and Salvucci, M.E.** (2002). Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. Annu Rev Plant Biol **53,** 449-475.

**Tang, W.H., Shilov, I.V., and Seymour, S.L.** (2008). Nonlinear fitting method for determining local false discovery rates from decoy database searches. J Proteome Res.

**Travis, A.J., and Mansfield, T.A.** (1979). Stomatal responses to light and $CO_2$ are dependent on KCI concentration. Plant, Cell and Environment **2,** 319-323.

**Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M.** (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science **292,** 2070-2072.

**Xie, Z., Ruas, P., and Shen, Q.J.** (2005). Regulatory networks of the phytohormone abscisic acid. Vitam Horm **72,** 235-269.

**Zhang, W., Qin, C., Zhao, J., and Wang, X.** (2004). Phospholipase Dα 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci U S A **101,** 9508-9513.

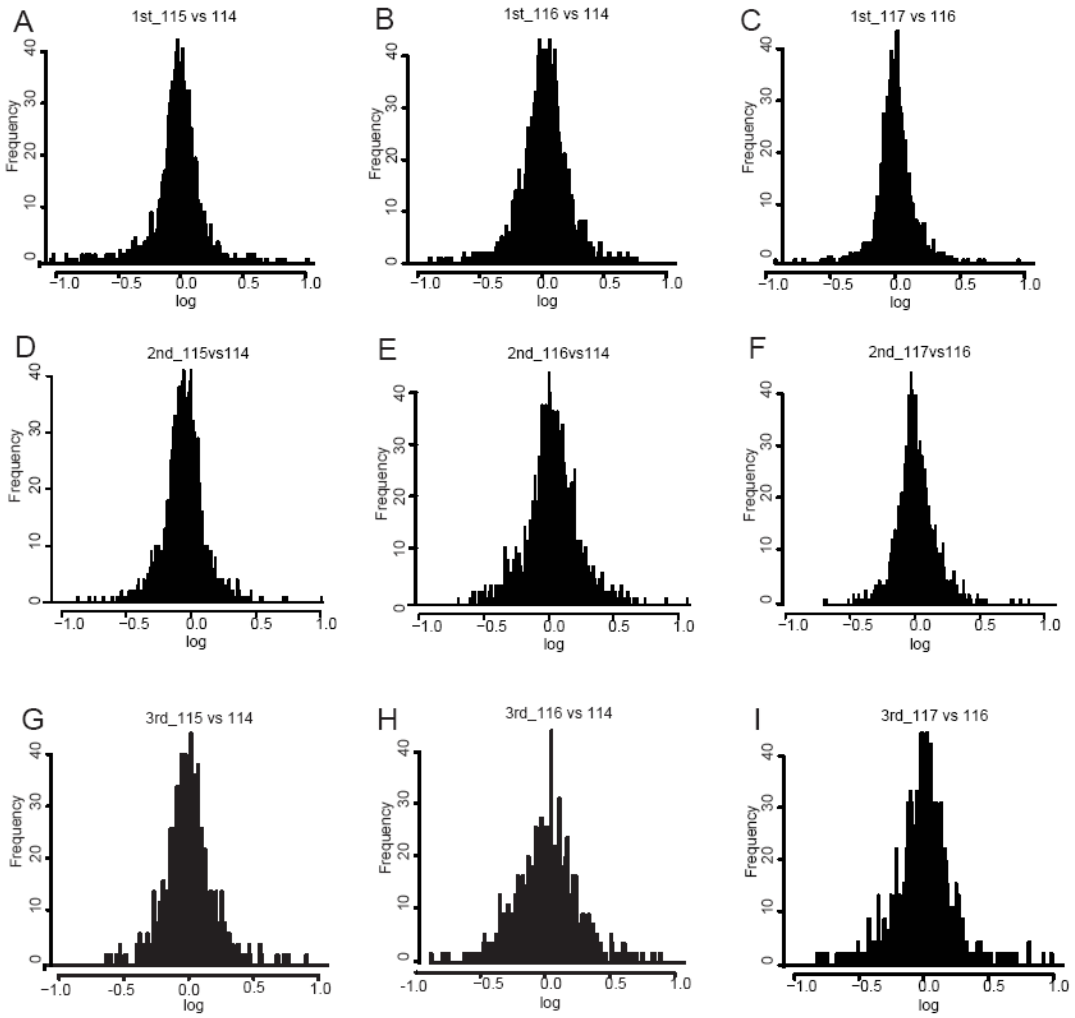



**Figure 3-1. No bias was introduced via the iTRAQ labeling reactions: all histograms are similar.**

A, B & C: The histogram of peptide ratios for the 1st iTRAQ experiment (A: 115:114, Col ABA vs Col, B: 116:114, *gpa1-4* vs Col, C: 117:116,*gpa1-4* ABA vs *gpa1-4*).
D, E & F: The histogram of peptide ratios for the 2nd iTRAQ experiment (D: 115:114, Col ABA vs Col, E: 116:114, *gpa1-4* vs Col, F: 117:116,*gpa1-4* ABA vs *gpa1-4*).
G, H & I: The histogram of peptide ratios for the 3rd iTRAQ experiment (G: 115:114, *gpa1-4* ABA vs *gpa1-4*, H: 116:114, Col vs *gpa1-4*, I: 117:116, Col ABA vs Col).

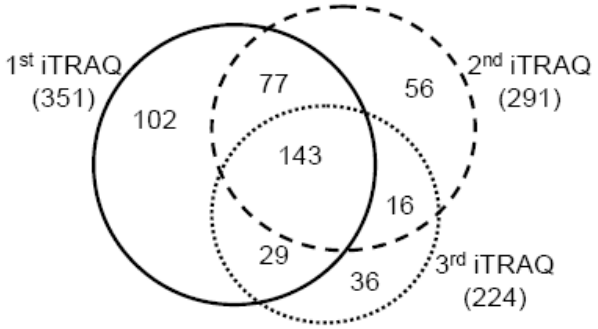



**Figure 3-2. The overlap in identified proteins between any two iTRAQ experiments is 49-71%.**
For the 1st, 2nd and 3rd iTRAQ experiment, 351, 291 and 224 proteins were identified respectively.

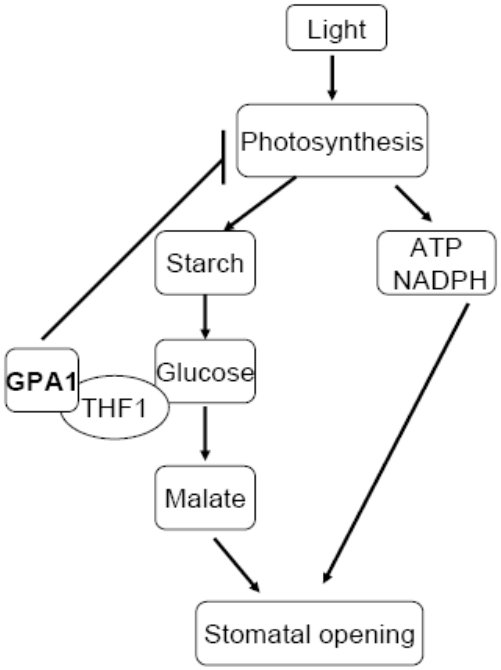



**Figure 3-3. A speculative model of GPA1 function in inhibiting photosynthesis in Arabidopsis guard cells.**

GPA1 is proposed to negatively regulate photosynthesis in guard cells by suppression of the abundances of select proteins of photophosphorylation and the Calvin cycle. Because GPA1 is known to interact with the plastid membrane protein THF1 and this interaction is proposed to function in glucose sensing (Huang et al., 2006), feedback inhibition of photosynthetic protein abundance and thus of guard cell photosynthesis might occur via GPA1-dependent sensing of the glucose concentration. Energy (ATP) and reducing power (NADPH) generated via photophosphorylation in guard cells contribute to the energetic requirements for light-stimulated stomatal opening (Schwartz and Zeiger, 1984). Starch hydrolysis produces glucose, which can be further hydrolyzed into malate precursors. Malate is known to be an important osmoticum during light-stimulated stomatal opening. → indicates that the pathway is activated by GPA1. ⊣ indicates that the pathway is inhibited by GPA1.

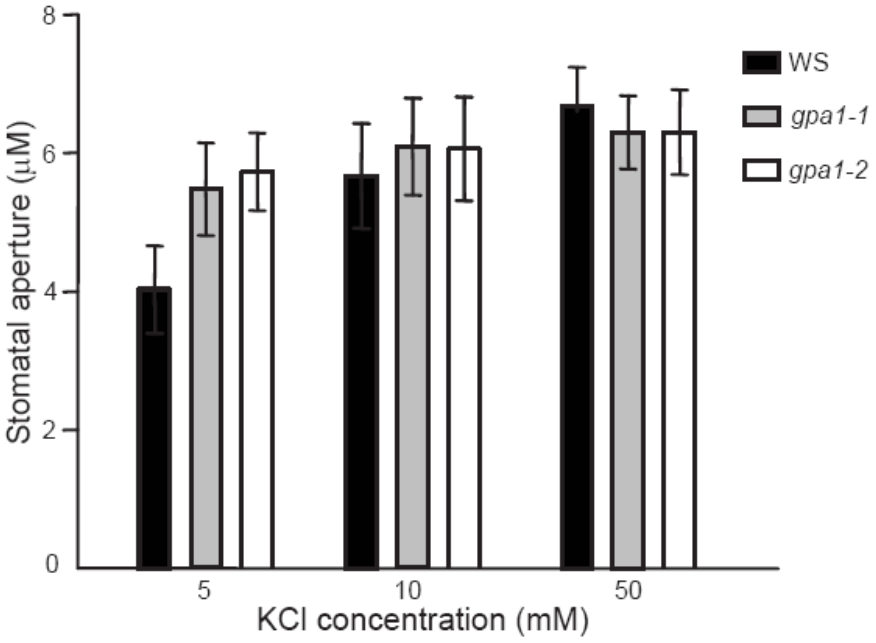



**Figure 3-4. Light promotion of stomatal opening is enhanced at lower $K^+$ concentrations in *gpa1* mutants (*gpa1-1* and *gpa1-2*).**

Light promotion of stomatal opening is positively related to the $K^+$ concentration of the incubation solution in both wild-type WS and *gpa1* plants, however, stomatal apertures are more open in *gpa1* mutants compared to the wild-type uner low concentration of $K^+$.

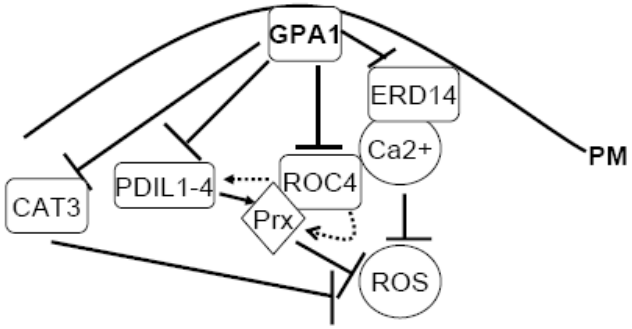



**Figure 3-5. A model of GPA1 function in promoting ROS concentration in Arabidopsis guard cells.**

GPA1 is proposed to positively regulate ROS concentration via down-regulating ERD14, CAT3 and ROC4 proteins. CAT3 is a $H_2O_2$ scavengers and decrease the intracellular concentration of $H_2O_2$ via conversion into water and oxygen (Frugoli et al., 1996). ROC4 can interact with peroxiredoxins (Prx) and regenerate the active form of Prx. Prx, similar to CAT3, are enzymes that decompose $H_2O_2$ (Dietz et al., 2006). The PDI activity of PDIL1-4 is increased in the presence of the PPI activity from ROC4. Therefore, PDIL1-4 may work together with ROC4 to regulate the correct folding of Prx, then further regulates the intracellular ROS concentration. ERD14 has membrane- (Kovacs et al., 2008) and $Ca^{2+}$- (Alsheikh et al., 2003) binding activity. By Sequestering the intracellular $Ca^{2+}$, ERD14 may further inhibit the production of ROS. .→ indicates up-regulation in protein amount. ⊣ indicates down-regulation in protein amount. ⇢ indicates up-regulation of the activity.

**Table 3-1. Two hundred and forty-nine proteins were identified in Arabidopsis leaves in our iTRAQ study.**

Proteins in bold were not identified previously by lee et al. (2007) and Baerenfaller et al. (2008).

| | | | | | | |
|---|---|---|---|---|---|---|
| At1g03475 | At1g52400 | At2g20260 | At3g14420 | At4g11010 | At5g14040 | AtCG00280 |
| At1g04410 | At1g53240 | At2g21170 | At3g15360 | At4g12420 | At5g14740 | AtCg00340 |
| At1g06680 | At1g53830 | At2g21330 | At3g15730 | At4g12800 | At5g16970 | AtCg00350 |
| At1g07890 | At1g54410 | At2g21660 | At3g16530 | At4g13940 | At5g17920 | AtCg00480 |
| At1g07930 | At1g54780 | At2g23600 | At3g16640 | At4g14880 | At5g19220 | AtCg00490 |
| At1g09210 | At1g55480 | At2g24270 | At3g17390 | At4g17390 | At5g19550 | AtCg00540 |
| At1g09340 | At1g55490 | At2g24820 | At3g18080 | At4g20260 | At5g19770 | AtCG00580 |
| At1g09750 | At1g55670 | At2g26080 | At3g19170 | At4g20360 | At5g20630 | AtCg00680 |
| At1g10760 | At1g56070 | At2g27710 | At3g23400 | At4g21860 | At5g20720 | **AtMg01190** |
| At1g11860 | At1g61520 | At2g30950 | At3g26650 | At4g22690 | At5g23060 | |
| At1g12900 | At1g62750 | At2g33150 | At3g28220 | At4g23630 | At5g23120 | |
| At1g14810 | At1g63770 | At2g34430 | At3g44310 | At4g24190 | At5g25460 | |
| At1g15690 | At1g63940 | At2g35410 | At3g45140 | At4g24280 | At5g25980 | |
| At1g15820 | At1g65930 | At2g36530 | At3g46780 | At4g24770 | At5g26000 | |
| At1g18080 | At1g66970 | At2g37220 | At3g46970 | At4g27440 | At5g28540 | |
| At1g20010 | At1g67090 | At2g39730 | At3g47800 | At4g28750 | At5g35630 | |
| At1g20020 | At1g68560 | At2g40840 | At3g49010 | At4g29060 | At5g36790 | |
| At1g20260 | At1g70600 | At2g43030 | At3g49120 | At4g30920 | At5g37360 | |
| At1g20340 | At1g71500 | At2g43750 | At3g50820 | At4g33010 | **At5g38410** | |
| At1g20620 | At1g72150 | At2g43910 | At3g52150 | At4g33510 | At5g38430 | |
| At1g21670 | At1g72610 | At2g45470 | At3g52880 | At4g35090 | At5g39570 | |
| At1g23310 | At1g73880 | At2g45960 | At3g52930 | At4g37930 | At5g42270 | |
| At1g26630 | At1g76180 | At2g47390 | At3g53420 | At4g38740 | At5g43940 | |
| At1g29670 | At1g78820 | At3g01280 | At3g53460 | At4g38970 | At5g44130 | |
| At1g30230 | At1g78830 | At3g01500 | At3g54050 | At4g39260 | At5g46110 | |
| At1g30380 | At1g78850 | At3g02730 | At3g55440 | At4g39730 | At5g46800 | |
| At1g31330 | At1g78900 | At3g04120 | At3g55800 | At5g01530 | At5g48300 | |
| At1g32060 | At1g79040 | At3g04790 | At3g57610 | At5g02500 | At5g50850 | |
| At1g32470 | At1g79550 | At3g06050 | At3g59970 | At5g03630 | At5g50920 | |
| At1g33590 | At1g80480 | At3g07390 | At3g60750 | At5g04140 | At5g51970 | |
| At1g35680 | At2g05100 | At3g08530 | At3g62030 | At5g04590 | At5g52310 | |
| At1g41830 | At2g05380 | At3g08580 | At3g63140 | At5g06290 | At5g54270 | |
| At1g42970 | At2g05520 | At3g08940 | At3g63410 | At5g08280 | At5g56030 | |
| At1g43170 | At2g05710 | At3g09630 | At3g63490 | At5g08680 | At5g58140 | |
| At1g44575 | At2g13360 | At3g09820 | At4g01050 | At5g09660 | At5g62690 | |
| At1g47128 | At2g15620 | At3g12810 | At4g02770 | At5g09810 | At5g63310 | |
| At1g48030 | At2g16600 | At3g13920 | At4g04640 | At5g10760 | At5g66190 | |
| At1g49240 | At2g18020 | At3g13930 | At4g05320 | At5g11670 | At5g66570 | |
| At1g49750 | At2g18960 | At3g14210 | At4g09010 | At5g12860 | AtCg00120 | |
| At1g52000 | At2g19940 | At3g14415 | At4g10340 | At5g13980 | AtCG00270 | |

**Table 3-2. In total, 454 proteins were identified in Arabidopsis Col guard cells in our iTRAQ study.**

**A. 351 proteins were identified in the 1st iTRAQ experiment.**

| | | | | | | |
|---|---|---|---|---|---|---|
| At1g01620 | At1g48030 | At2g21160 | At3g12810 | At4g02510 | At4g38740 | At5g43330 |
| At1g02500 | At1g48920 | At2g21170 | At3g13570 | At4g02930 | At4g38970 | At5g43830 |
| At1g02780 | At1g49750 | At2g21390 | At3g13930 | At4g10340 | At4g39090 | At5g43940 |
| At1g04410 | At1g50010 | At2g21660 | At3g14210 | At4g11010 | At4g39260 | At5g44130 |
| At1g06680 | At1g50480 | At2g24200 | At3g15730 | At4g11420 | At4g39330 | At5g46110 |
| At1g07660 | At1g51980 | At2g24270 | At3g16470 | At4g12420 | At4g39660 | At5g46290 |
| At1g07670 | At1g52360 | At2g25970 | At3g16640 | At4g12800 | At4g39730 | At5g47690 |
| At1g07890 | At1g53210 | At2g26250 | At3g17240 | At4g13430 | At5g01530 | At5g48180 |
| At1g07940 | At1g53240 | At2g28190 | At3g17390 | At4g13930 | At5g02500 | At5g48480 |
| At1g08830 | At1g53500 | At2g30620 | At3g17820 | At4g13940 | At5g03740 | At5g49720 |
| At1g09210 | At1g54410 | At2g30950 | At3g18780 | At4g14360 | At5g04140 | At5g50000 |
| At1g09780 | At1g55490 | At2g30970 | At3g18890 | At4g14880 | At5g04430 | At5g50850 |
| At1g11260 | At1g56070 | At2g32670 | At3g19820 | At4g16660 | At5g04590 | At5g52040 |
| At1g11580 | At1g56340 | At2g33150 | At3g22960 | At4g17390 | At5g06530 | At5g54270 |
| At1g11650 | At1g58270 | At2g34430 | At3g23390 | At4g20360 | At5g07090 | At5g55160 |
| At1g11840 | At1g58380 | At2g36530 | At3g26430 | At4g20890 | At5g07350 | At5g55190 |
| At1g11860 | At1g61520 | At2g36580 | At3g26520 | At4g21150 | At5g07460 | At5g55660 |
| At1g11910 | At1g61790 | At2g36620 | At3g26650 | At4g22970 | At5g08690 | At5g56030 |
| At1g13440 | At1g62480 | At2g36880 | At3g27690 | At4g23630 | At5g09590 | At5g58710 |
| At1g15690 | At1g63940 | At2g37220 | At3g44110 | At4g24190 | At5g09810 | At5g60640 |
| At1g15820 | At1g64090 | At2g39730 | At3g44310 | At4g24280 | At5g11670 | At5g60980 |
| At1g16610 | At1g64370 | At2g39850 | At3g45980 | At4g24770 | At5g12250 | At5g61790 |
| At1g17840 | At1g64740 | At2g40060 | At3g47520 | At4g25630 | At5g14040 | At5g62390 |
| At1g18080 | At1g65790 | At2g40100 | At3g48990 | At4g26630 | At5g15090 | At5g62690 |
| At1g18540 | At1g65930 | At2g40840 | At3g49010 | At4g26970 | At5g15490 | At5g65750 |
| At1g20010 | At1g67090 | At2g41840 | At3g49430 | At4g27440 | At5g17920 | At5g66190 |
| At1g20020 | At1g67230 | At2g42600 | At3g49680 | At4g27500 | At5g19510 | At5g66400 |
| At1g20260 | At1g68530 | At2g43750 | At3g49720 | At4g28750 | At5g19550 | At5g66570 |
| At1g20340 | At1g70730 | At2g44160 | At3g50670 | At4g29350 | At5g19690 | At5g66680 |
| At1g20450 | At1g72150 | At2g45960 | At3g50820 | At4g30190 | At5g19770 | At5g66760 |
| At1g20620 | At1g72370 | At2g46070 | At3g51880 | At4g31340 | At5g20290 | At5g67500 |
| At1g20940 | At1g76180 | At2g47240 | At3g52590 | At4g31480 | At5g20290 | AtCg00020 |
| At1g21540 | At1g78370 | At3g01280 | At3g52880 | At4g31700 | At5g20720 | AtCg00120 |
| At1g21750 | At1g78850 | At3g01500 | At3g52930 | At4g31880 | At5g22650 | AtCg00270 |
| At1g23190 | At1g78900 | At3g03780 | At3g52960 | At4g32410 | At5g23120 | AtCg00280 |
| At1g23290 | At1g79040 | At3g04120 | At3g53420 | At4g33010 | At5g23250 | AtCg00340 |
| At1g23310 | At1g79550 | At3g04840 | At3g54050 | At4g33090 | At5g25100 | AtCg00350 |
| At1g24360 | At1g79920 | At3g05060 | At3g55360 | At4g33680 | At5g25980 | AtCg00480 |
| At1g26110 | At1g80070 | At3g06050 | At3g55440 | At4g34200 | At5g26000 | AtCg00490 |
| At1g26630 | At1g80480 | At3g06650 | At3g55460 | At4g34870 | At5g28540 | AtCg00540 |
| At1g26910 | At1g80930 | At3g07110 | At3g55800 | At4g34870 | At5g35160 | AtCg00680 |

| | | | | | | |
|---|---|---|---|---|---|---|
| At1g29470 | At2g05710 | At3g07390 | At3g57610 | At4g35100 | At5g35630 | AtCg00720 |
| At1g30360 | At2g05990 | At3g08580 | At3g58610 | At4g35260 | At5g36880 | AtMg00160 |
| At1g31330 | At2g14720 | At3g08940 | At3g59970 | At4g35630 | At5g37510 | AtMg01190 |
| At1g31850 | At2g16600 | At3g09440 | At3g60750 | At4g36130 | At5g38420 | AtMg01360 |
| At1g32060 | At2g18020 | At3g09630 | At3g62030 | At4g36250 | At5g39570 | |
| At1g32470 | At2g18960 | At3g09820 | At3g63410 | At4g37300 | At5g39740 | |
| At1g42970 | At2g19520 | At3g09840 | At3g63460 | At4g37910 | At5g42020 | |
| At1g43170 | At2g19760 | At3g10400 | At4g00360 | At4g37930 | At5g42270 | |
| At1g44575 | At2g20760 | At3g11130 | At4g00400 | At4g38350 | At5g42790 | |
| At1g47128 | At2g20990 | At3g11250 | At4g02450 | At4g38680 | At5g42820 | |
| **B. 291 proteins were identified in the 2nd iTRAQ experiment** | | | | | | |
| At1g01610 | At1g44575 | At2g20260 | At3g13920 | At4g00360 | At4g37870 | At5g37510 |
| At1g01620 | At1g47128 | At2g20990 | At3g13930 | At4g01050 | At4g37930 | At5g38410 |
| At1g02780 | At1g48030 | At2g21170 | At3g14067 | At4g01850 | At4g38510 | At5g39570 |
| At1g04410 | At1g48600 | At2g21390 | At3g15730 | At4g02450 | At4g38740 | At5g39740 |
| At1g06680 | At1g48850 | At2g21660 | At3g16640 | At4g02930 | At4g38970 | At5g40340 |
| At1g07660 | At1g48920 | At2g24200 | At3g17390 | At4g10340 | At4g39330 | At5g42020 |
| At1g07750 | At1g50010 | At2g24270 | At3g17820 | At4g11420 | At4g39730 | At5g42270 |
| At1g07890 | At1g51980 | At2g24420 | At3g18780 | At4g12420 | At5g01530 | At5g43060 |
| At1g07940 | At1g53210 | At2g25970 | At3g19820 | At4g12800 | At5g02500 | At5g43830 |
| At1g08830 | At1g53240 | At2g26080 | At3g22960 | At4g13430 | At5g03740 | At5g43940 |
| At1g09210 | At1g54410 | At2g26250 | At3g24830 | At4g13850 | At5g04140 | At5g46290 |
| At1g09780 | At1g56070 | At2g28190 | At3g26520 | At4g13930 | At5g04590 | At5g47210 |
| At1g10950 | At1g56340 | At2g29550 | At3g26650 | At4g13940 | At5g05010 | At5g47690 |
| At1g11260 | At1g59359 | At2g30620 | At3g28860 | At4g14040 | At5g07350 | At5g48180 |
| At1g11840 | At1g59870 | At2g33150 | At3g43300 | At4g14880 | At5g07460 | At5g49460 |
| At1g11860 | At1g61520 | At2g36530 | At3g44310 | At4g16660 | At5g08300 | At5g54270 |
| At1g11910 | At1g61790 | At2g36880 | At3g48730 | At4g17390 | At5g08450 | At5g55660 |
| At1g12900 | At1g63940 | At2g37220 | At3g49010 | At4g18030 | At5g08670 | At5g56030 |
| At1g13440 | At1g64370 | At2g39730 | At3g49430 | At4g20360 | At5g09810 | At5g57290 |
| At1g15690 | At1g65930 | At2g39850 | At3g50670 | At4g20890 | At5g10010 | At5g58420 |
| At1g15820 | At1g67090 | At2g41840 | At3g50820 | At4g21150 | At5g10360 | At5g58710 |
| At1g17840 | At1g68530 | At2g42600 | At3g52590 | At4g23630 | At5g11560 | At5g60640 |
| At1g18080 | At1g70730 | At2g43030 | At3g52880 | At4g23900 | At5g14040 | At5g61790 |
| At1g18540 | At1g70770 | At2g45960 | At3g52930 | At4g24190 | At5g15090 | At5g62390 |
| At1g20010 | At1g74960 | At2g46070 | At3g52960 | At4g24280 | At5g15350 | At5g64030 |
| At1g20020 | At1g76180 | At2g47240 | At3g53420 | At4g24770 | At5g17920 | At5g66190 |
| At1g20340 | At1g76810 | At2g47510 | At3g53520 | At4g26630 | At5g19510 | At5g66570 |
| At1g20620 | At1g78360 | At3g01280 | At3g53720 | At4g26970 | At5g19690 | At5g66760 |
| At1g20960 | At1g78850 | At3g01500 | At3g54050 | At4g27500 | At5g19770 | AtCg00020 |
| At1g21540 | At1g78900 | At3g03780 | At3g55410 | At4g28750 | At5g20010 | AtCg00120 |
| At1g21750 | At1g79040 | At3g07390 | At3g55440 | At4g29010 | At5g20290 | AtCg00270 |
| At1g23190 | At1g79550 | At3g08530 | At3g55460 | At4g31490 | At5g20720 | AtCg00280 |
| At1g23290 | At1g79920 | At3g08580 | At3g55800 | At4g31880 | At5g22650 | AtCg00340 |
| At1g26630 | At1g80070 | At3g08940 | At3g57150 | At4g33010 | At5g22880 | AtCg00350 |

| | | | | | | |
|---|---|---|---|---|---|---|
| At1g29470 | At1g80300 | At3g09440 | At3g57410 | At4g33680 | At5g23010 | AtCg00480 |
| At1g29910 | At2g01140 | At3g09630 | At3g57610 | At4g34200 | At5g25980 | AtCg00490 |
| At1g30360 | At2g04030 | At3g09820 | At3g58610 | At4g34450 | At5g26000 | AtCg00540 |
| At1g31330 | At2g05710 | At3g09840 | At3g59970 | At4g34870 | At5g26000 | AtCg00680 |
| At1g32060 | At2g05990 | At3g12390 | At3g60750 | At4g35630 | At5g27850 | AtMg00160 |
| At1g32470 | At2g16600 | At3g12810 | At3g62030 | At4g35830 | At5g28540 | AtMg01190 |
| At1g42970 | At2g18960 | At3g13470 | At3g63140 | At4g36130 | At5g34850 | |
| At1g43170 | At2g19520 | At3g13570 | At3g63410 | At4g36250 | At5g35630 | |
| **C. 224 proteins were identified in the 3rd iTRAQ experiment.** | | | | | | |
| At1g01620 | At1g48920 | At2g21090 | At3g12810 | At4g12420 | At5g02500 | At5g39740 |
| At1g02780 | At1g50250 | At2g21160 | At3g13570 | At4g12650 | At5g03300 | At5g40890 |
| At1g04410 | At1g51980 | At2g21170 | At3g13930 | At4g12800 | At5g04430 | At5g42020 |
| At1g06680 | At1g52360 | At2g21660 | At3g15730 | At4g13770 | At5g04590 | At5g42790 |
| At1g07940 | At1g53240 | At2g24270 | At3g16470 | At4g13850 | At5g05010 | At5g43830 |
| At1g08830 | At1g54410 | At2g24420 | At3g17390 | At4g14960 | At5g06530 | At5g43940 |
| At1g09210 | At1g54690 | At2g25970 | At3g18035 | At4g17390 | At5g07460 | At5g44130 |
| At1g09780 | At1g56070 | At2g26080 | At3g18780 | At4g20360 | At5g08300 | At5g46110 |
| At1g11260 | At1g56340 | At2g26250 | At3g19820 | At4g20890 | At5g08690 | At5g46750 |
| At1g11840 | At1g58380 | At2g28190 | At3g26520 | At4g23630 | At5g09590 | At5g47690 |
| At1g11860 | At1g63940 | At2g29550 | At3g26650 | At4g24280 | At5g09810 | At5g49760 |
| At1g11910 | At1g65930 | At2g30620 | At3g27690 | At4g24770 | At5g12250 | At5g54270 |
| At1g13280 | At1g67090 | At2g30950 | At3g42170 | At4g25130 | At5g14030 | At5g55160 |
| At1g13440 | At1g68530 | At2g33410 | At3g42640 | At4g26630 | At5g14040 | At5g55660 |
| At1g14820 | At1g69620 | At2g34430 | At3g44310 | At4g27000 | At5g15090 | At5g56030 |
| At1g15820 | At1g70730 | At2g36530 | At3g49010 | At4g29350 | At5g15350 | At5g57290 |
| At1g20010 | At1g74060 | At2g37220 | At3g49430 | At4g31490 | At5g19690 | At5g60640 |
| At1g20340 | At1g76180 | At2g38550 | At3g52590 | At4g31580 | At5g19770 | At5g62200 |
| At1g20620 | At1g78850 | At2g39730 | At3g52880 | At4g31700 | At5g20290 | At5g62690 |
| At1g21540 | At1g78900 | At2g41840 | At3g53420 | At4g31880 | At5g20720 | At5g66570 |
| At1g21750 | At1g79040 | At2g43750 | At3g54050 | At4g34200 | At5g22650 | AtCg00020 |
| At1g23290 | At1g79550 | At2g45960 | At3g55360 | At4g34490 | At5g22880 | AtCg00120 |
| At1g26110 | At1g79930 | At3g01500 | At3g55440 | At4g34870 | At5g23120 | AtCg00270 |
| At1g29370 | At1g80930 | At3g03780 | At3g55800 | At4g35100 | At5g25980 | AtCg00280 |
| At1g29470 | At2g04030 | At3g04120 | At3g57150 | At4g35630 | At5g26000 | AtCg00340 |
| At1g30360 | At2g16600 | At3g08530 | At3g58610 | At4g38510 | At5g27850 | AtCg00350 |
| At1g31330 | At2g17120 | At3g08580 | At3g59970 | At4g38680 | At5g28540 | AtCg00480 |
| At1g32060 | At2g18020 | At3g09440 | At3g60750 | At4g38740 | At5g28780 | AtCg00490 |
| At1g42970 | At2g18960 | At3g09630 | At3g62030 | At4g38780 | At5g34850 | AtCg00540 |
| At1g43170 | At2g19520 | At3g09820 | At3g63410 | At4g39330 | At5g35630 | AtCg00680 |
| At1g44575 | At2g19750 | At3g09840 | At4g02450 | At4g39650 | At5g38420 | AtCg00720 |
| At1g47128 | At2g20760 | At3g10740 | At4g11420 | At5g01530 | At5g39570 | AtCg01060 |

**Table 3-3. Eighteen proteins were identified to be quantitatively affected by null mutation of *GPA1* in guard cells.**

| AGI | Sub | BP | R1 | P1 | R2 | P2 | R3 | P3 | Name |
|---|---|---|---|---|---|---|---|---|---|
| AtCg00120 | C | ATP synthase | 1.44 | 0.01 | 1.42 | 0.01 | | | ATP synthase CF1 alpha subunit |
| AtCg00480 | C | ATP synthase | 1.28 | 0.01 | 1.42 | 0.01 | | | ATPB |
| At1g42970 | C | Calvin cycle; glycolysis | 1.25 | 0.01 | 1.21 | 0.01 | | | GAPB |
| At1g67090 | C | Calvin cycle | 1.87 | 0.01 | 1.54 | 0.02 | 2.05 | 0.02 | Rubisco small subunit |
| At2g39730 | C | Calvin cycle | | | 1.70 | 0.02 | 2.19 | 0.04 | RAC |
| At3g55800 | C | Calvin cycle | 1.34 | 0.03 | 1.58 | 0.01 | | | SBPase |
| At3g60750 | C | Calvin cycle | | | 1.33 | 0.01 | 1.56 | 0.01 | transketolase-like protein |
| AtCg00490 | C | Calvin cycle | 1.43 | 0.01 | 1.46 | 0.01 | 1.44 | 0.01 | RBCL |
| At1g06680 | C | Photosynthesis, light reaction | 1.29 | 0.01 | 1.40 | 0.01 | | | PsbP1 |
| At1g79040 | C | Photosynthesis, light reaction | 1.51 | 0.05 | 1.58 | 0.01 | | | PsbR |
| At5g66570 | C | Photosythesis, light reaction | 1.54 | 0.01 | | | 1.80 | 0.03 | PSBO1 |
| At1g20340 | C | Photosynthesis, Lumenal electron carrier | 1.81 | 0.01 | 2.22 | 0.01 | | | PETE2/ DRT112 |
| At1g20620 | C, M | Response to stress | 1.36 | 0.01 | 1.46 | 0.01 | 1.45 | 0.01 | CAT3 |
| At1g76180 | C | Response to stress | 1.59 | 0.01 | 2.01 | 0.01 | 2.84 | 0.04 | ERD14 |
| At3g62030 | C | Response to stress | 1.53 | 0.01 | 1.42 | 0.01 | | | ROC4 (CYP20-3) |
| At5g25980 | C ER | Response to stress | 1.44 | 0.01 | 1.46 | 0.01 | 1.72 | 0.01 | TGG2 |
| At5g60640 | C, M | Response to stress, Protein folding | 1.25 | 0.05 | 1.59 | 0.04 | | | PDIL1-4 |
| At5g55660 | M | Unknown | **0.71** | 0.04 | **0.58** | 0.02 | | | Unknown protein |

AGI: The gene identifier from the Arabidopsis Genome Initiative. Sub: subcellular localization. BP: biological process. C. chloroplast, M. mitochondrion, ER. endoplasmic reticulum. R: Replicate. Numbers in the R columns are iTRAQ ratios of *gpa1-4*/Col, and the decreased ratio is in bold. P: The calculated P value for the corresponding iTRAQ ratio.
The predicted subcellular localization and biological process were predicted by the GO software at the TAIR website: www.arabidopsis.org.

**Table 3-4. Eight proteins were identified to be significantly regulated by ABA in protein abundance in guard cells**.

**a. Two proteins were significantly affected by ABA in protein abundance in Col guard cells.**

| AGI | Sub | BP | R1 | P1 | R2 | P2 | R3 | P3 | Name |
|---|---|---|---|---|---|---|---|---|---|
| At1g53240 | M | Response to stress | | | 1.25 | 0.01 | 1.48 | 0.02 | MDH |
| At5g38420 | C | Calvin cycle | 1.17 | 0.05 | | | 1.19 | 0.04 | rbcS2B |

**b. Six proteins were significantly affected by ABA in protein abundance in *gpa1-4* guard cells.**

| AGI | Sub | BP | R1 | P1 | R2 | P2 | R3 | P3 | Name |
|---|---|---|---|---|---|---|---|---|---|
| AtCg00280 | C | Photosynthesis, light reaction | 1.65 | 0.01 | 1.51 | 0.01 | | | PsbC |
| AtCg00340 | C | Photosynthesis, light reaction | 1.26 | 0.02 | 1.58 | 0.01 | 1.61 | 0.01 | PsaB |
| AtCg00350 | C | Photosynthesis, light reaction | 1.41 | 0.01 | 1.37 | 0.01 | 1.56 | 0.01 | PsaA |
| AtCg00680 | C | Photosynthesis, light reaction | 1.34 | 0.01 | 1.3 | 0.01 | 1.53 | 0.01 | PsbB |
| At1g15820 | C | Photosynthesis, PSII antennae | **0.76** | 0.01 | **0.72** | 0.01 | | | lhcb6/CP24, |
| At1g76180 | C | Response to stress | | | **0.78** | 0.02 | **0.72** | 0.02 | ERD14 |

AGI: The protein identifier named by Arabidopsis Genome Initiative. Sub: subcellular localization. BP: biological process. C. chloroplast, M. mitochondrion. R: Replicate. Numbers in the R columns are iTRAQ ratios of Col ABA/Col and *gpa1-4* ABA/*gpa1-4* in section a and b, respectively, and the decreased ratios are in bold. P: The calculated P value for the corresponding iTRAQ ratio.

The predicted subcellular localization and biological process were predicted by the GO software at the TAIR website: www.arabidopsis.org .

**Table 3-5. A summary of criteria used in the published literature on iTRAQ studies in plants.**

| Searching algorithm | Protein confidence | P | Criteria for fold change of the identified proteins | Reference |
|---|---|---|---|---|
| Mascot | 94% | P<0.05 | Any protein identified in two replicates. | (Jones et al., 2006) |
| Mascot | Protein score>20 | P<0.05 | Any protein identified in two replicates. | (Kaffarnik et al., 2008) |
| Mascot | FDR<1% | NA | Proteins with fold change < 0.85 or > 1.15, and identified in two replicates. | (Rutschow et al., 2008) |
| Mascot | 95% | NA | NA | (Majeran et al., 2008) |
| Protein Pilot | 99.99% | P<0.05 | Protein with fold change < 0.85 or > 1.17, and identified in two replicates. | Our Data |

FDR: False discovery rate. NA: not available. P: signifies the calculated P value for the identified iTRAQ ratios.

**Table 3-6. Protein abundances of four proteins identified in our iTRAQ study and known to be involved in ABA signaling in guard cells, were not identified to be quantitatively affected by ABA in guard cells in our study.**

| Protein name | AGI | Phenotype of the knock-out mutants | Reference |
|---|---|---|---|
| PLDα1 | At3g15730 | Insensitive to ABA regulation of stomatal movements. | (Mishra et al., 2006) |
| CHX20 | At3g53720 | Delayed in ABA-induced stomatal closure.. | (Padmanaban et al., 2007) |
| OST2 | At2g18960 | Hyposensitive to ABA promotion of stomatal closure | (Merlot et al., 2007) |
| TGG1 | At5g26000 | Insensitive to ABA inhibition of stomatal opening | (Zhao et al., submitted) |

# Chapter 4

## The correlation between the transcriptome and the proteome of Arabidopsis guard cells

In this chapter, the RNA isolation, cDNA and cRNA synthesis were performed by Dr. Sona Pandey, the microarray analysis data and Figure 4-4 were provided by Song Li. All other results were generated by Zhixin Zhao.

**Abstract**

The mRNA abundance of a transcript is often used as a proxy for the protein abundance, presumably due to the relationship between these two types of molecules in the central dogma. However, to our knowledge, the correlation between mRNA and protein levels has not been studied in a single cell type of plants to date. In this analysis, the correlation between the transcriptome and the proteome was studied in a single cell type: the Arabidopsis guard cell. Global transcriptomic data were obtained using microarrays, and proteomic data were obtained via gel-based and gel-free (MUltiDimensional Protein Identification Technology; MudPIT) methods. A comparison of the transcriptomic and the proteomic data revealed poor correlation between mRNA and protein levels in Arabidopsis guard cells. Further analysis using subsets of the identified proteins showed that the extent of correlation between the transcriptome and proteome in guard cells differs according to protein function and subcellular localization.

**Introduction**

With the latest advances in high throughput genomic and proteomic techniques, thousands of genes or proteins can be detected and quantified simultaneously. Large-scale datasets from these kinds of research efforts are valuable and necessary for understanding complex biological processes in a given organism. According to the central dogma of molecular biology, a stretch of the genomic deoxyribonucleic acid (DNA), or a gene, is transcribed into a messenger ribonucleic acid (mRNA), and the mRNA is in turn translated into a protein (or proteins). In a given organism, the genome contains the entire genetic information in the form of DNA and remains relatively stable in comparison to the proteome. A proteome refers to the sum of all proteins produced in a given cell, tissue or organism under a certain condition. Many factors play roles during

the mRNA translation process such as alternative splicing, RNA silencing, protein degradation, and mRNA and protein turnover rate. Therefore, multiple proteins can be synthesized from one mRNA and a single genome can generate myriad proteomes (Tyers and Mann, 2003).

The abundance of an mRNA is often considered as a reasonable indicator of the level of its corresponding protein product. Although mRNA levels can be easily monitored using common techniques such as Northern blotting and reverse transcription-polymerase chain reaction (RT-PCR) methods, protein levels are more difficult to determine because proteins are more difficult to purify and proteins cannot be amplified with current technologies. Researchers often estimate changes in quantity and thus function of a protein by its mRNA levels, assuming a good correlation exists between mRNA and protein levels.

Global comparison of a proteome and its corresponding transcriptome to test this assumption has become feasible by application of high throughput transcript profiling technologies and quantitative proteomic technologies. These methods include serial analysis of gene expression (SAGE) (Velculescu et al., 1995), massively parallel signature sequencing (MPSS) (Brenner et al., 2000; Reinartz et al., 2002), microarray analysis (Valor and Grant, 2007), and the next-generation sequencing methods: 454 sequencer (Margulies et al., 2005), Solexa (Barski et al., 2007), Illumina genome analyzer and Sequencing by Oligo Ligation and Detection (SOLiD) (Mardis, 2008) for transcriptomic studies, and blue-native gel analysis (Schagger et al., 1994), two dimensional (2D) gel electrophoresis analysis (Berkelman, 2008), LC-MS/MS (Ishihama et al., 2005), Isotope-Coded Affinity Tag (ICAT) (Gygi et al., 1999b) and Isotope Tags for Relative and Absolute Quantification (iTRAQ) (Schneider and Hall, 2005) for proteomic studies.

Each single cell type in a multicellular organism has unique functions reflecting its location and role in the organism (Immink et al., 2003). Due to the difficulty of obtaining a sufficient quantity of highly pure cells of a single cell type, proteomic studies of single cell types isolated from a multicellular organism are still in their infancy. Comparison of the transcriptome and proteome of single cell types can provide more

accurate information about the relationship between mRNA and protein levels than tissue or organ level comparisons, and can facilitate understanding of the protein regulatory mechanisms operating in that particular cell type. However, no such comparison has been made in plants to date.

Microscopic holes in the surfaces of leaves are called stomatal pores and each pore is regulated by a pair of cells called guard cells. Stomata affect photosynthesis and transpiration by regulating stomatal pore apertures, through which carbon dioxide ($CO_2$) uptake and water vapor loss occur. As a unique cell type in plants, the guard cell is also a popular model system in plant cell biology (Schroeder et al., 2001b; Fan et al., 2004; Li et al., 2006). To evaluate the correlation between the transciptome and the proteome, the guard cell transcriptome was examined by microarray methodology, and the proteome of guard cells was obtained by two gel-based methods, broad pH range 2D gels (BR) and narrow pH range 2D gels (NR), and one gel-free method, Liquid Chromatography (LC) - Matrix-Assisted Laser-Desorption Ionization (MALDI) MUltiDimensional Protein Identification Technology (MudPIT) (Chapter 2). Comparison of transcriptomic and proteomic data revealed an overall poor correlation between mRNA and protein abundance in Arabidopsis guard cells, although the correlation coefficient between mRNA and protein levels in guard cells was improved for certain GO categories of protein function or subcellular localization.

The heterotrimeric G protein $\alpha$ subunit, GPA1, positively modulates ABA signaling in Arabidopsis guard cells (Wang et al., 2001; Fan et al., 2008). Transcripts regulated by null mutation of *GPA1* and by ABA treatment of guard cells were quantified using Affymetrix ATH1 Arabidopsis microarrays and proteins quantitatively regulated by *GPA1* and ABA were identified using the iTRAQ method (Chapter 3). Comparison between the transcriptome and the proteome suggested poor co-regulation of these –omes by *GPA1* or ABA in Arabidopsis guard cells.

## Results

Transcriptomic and proteomic data from Arabidopsis guard cells were used to study the global correlation between mRNA and protein levels. As previously reported, 1764 proteins were identified from WS guard cells from the combined application of three proteomic methods (Chapter 2). To examine the correlation of mRNA and protein levels in guard cells, microarray analysis using Affymetrix ATH1 chips, each chip containing 22,500 probes that represent about 24,000 genes, was performed using RNA extracted from WS guard cell protoplasts (GCPs). To be consistent, the GCPs were isolated exactly the same way as for the proteomic studies (Chapter 2). The expression value was obtained for each transcript present in the transcriptome, and the average of the expression values of each transcript from three independent biological replicates was used.

### Comparison of guard cell proteome and transcriptome

Transcripts encoding 168 proteins were found to have probes in common with other genes. For example, genes such as At5g26000 (*TGG1*) and At5g29800 (*TGG2*) share the same probe, 246880_s_at, on the microarray chip. Transcripts encoding 19 proteins were represented by multiple probes on the microarray chip. Transcripts encoding 46 proteins identified in our guard cell proteome (two, At1g16140 and At2g46320, from the NR method and 44 from the LC-MALDI MudPIT method) were not represented by probes on the microarray chip. All these transcripts and proteins encoded by these transcripts were eliminated for calculating the correlation coefficients between mRNA and protein abundance.

It has been shown previously that it is more difficult to identify smaller proteins using shotgun proteomics (Liu et al., 2004; Baerenfaller et al., 2008). In our study, predicted molecular weights of proteins which were represented in our transcriptome, but not identified by proteomic methods were compared to the predicted molecular weights

of all the identified proteins from one or two replicates of the LC-MALDI MudPIT method using Mascot or Protein Pilot software (**Figure 4-1**). Of transcripts that were present in the transcriptome but did not have corresponding proteins in the proteome, a greater percentage of these encoded small molecular mass proteins, as compared to the molecular masses of all the proteins identified by LC-MALDI MudPIT method (**Figure 4-1**). For example, 3% and 13% of the proteins that were represented in the transcriptome but not in the actual proteome have molecular masses less than 10 kD and 10-20 kD respectively, however, only 0.5% and 7% of all the proteins identified by the LC-MALDI MudPIT method have molecular masses less than 10 kD and 10-20 kD respectively (**Figure 4-1**). Therefore, our data support the notion that smaller proteins tend to be missed by the shotgun method.

Transcriptome and proteome comparison revealed that 117 proteins (112 of the 117 proteins from the LC-MALDI MudPIT method and five of the 117 proteins from the NR method) were absent at the transcriptional level but were identified in our proteomic study (**Table 4-1**). The relative protein abundance of these 112 LC-MALDI MudPIT identified proteins was further compared to that of all 1,742 proteins identified from the LC-MALDI MudPIT method in one or two replicates using Mascot or Protein Pilot software (Chapter 2). Of the 112 proteins, 25.9% were identified by only one peptide, suggestive of low abundance, whereas only 15% of the 1,742 proteins identified by the LC-MALDI MudPIT method were identified by one peptide (**Figure 4-2**). This indicates that transcripts of low abundance proteins are more likely to be absent from the transcriptome. The GO (http://www.arabidopsis.org/tools/bulk/go/index.jsp) distribution pattern of the 112 proteins was similar to that of the 1,742 proteins identified from the LC-MALDI MudPIT method (data not shown).

On the basis of mRNA levels, the whole guard cell transcriptome was divided into six abundance categories (Log2=2-4, 4-6, 6-8, 8-10, 10-12, >12). The number of transcripts encoding identified guard cell proteins in each category was calculated and compared to the total number of transcripts in that category (**Table 4-2**). Our data showed that proteins encoded by 18.2% of transcripts in the highest abundance category (Log2>12) were identified by proteomic methods, however, the percentage dropped to

only 0.8% in the lowest transcript abundance category (Log2=2-4) (**Table 4-2**). These results indicate that proteins with highly abundant transcripts are more likely to be present in the proteome. Thus, on the basis of the broad categories of presence or absence, there does appear to be a correlation between transcriptomic and proteomic data. However, as described below, for proteins represented in both the proteome and the transcriptome, there is little to no correlation in transcript and protein abundance.

**Quantitation of protein abundance**

The spots corresponding to all the identified proteins in the gel-based methods (Chapter 2) were quantitatively analyzed by PDQuest software according to spot intensities. The absolute protein amount in each spot is represented by the spot quantity - the integrated intensity of the spot. Gels from two independent biological replicates were used for spot-cutting, trypsin digestion and protein identification (Chapter 2), and only protein spots detected in both replicate gels were excised. The average protein amount from the two replicates for each spot was quantified by the PDQuest software. All spots in which more than one protein was identified from the spot were eliminated from analysis because it was not possible to determine the protein amount for each protein in the spot. In total, 46 proteins from the BR and 47 proteins from the NR method were used for comparison to their corresponding mRNA levels. The correlation coefficient using proteins identified either from BR (**Figure 4-3A**) or NR (**Figure 4-3B**) was -0.06.

The relative protein abundance in one MudPIT sample can be reflected by the number of identified unique peptides derived from that protein in the shotgun method (Corbin et al., 2003). Just as for the gel-based methods, also for the LC-MALDI MudPIT method only proteins identified in two independent biological replicates were included in the analysis, and protein abundances were averaged. It has been shown that spectral count is a more accurate method to measure relative protein abundance (Liu et al., 2004), however, the spectra obtained by the LC-MALDI MudPIT method in our study may be not valid for measurement by the spectra counting method. The main reason is that a

strict dynamic exclusion is applied in the LC-MALDI MudPIT method and only one ms/ms spectrum per m/z in each MALDI plate was reported and then the rest of the elution gradient is ignored, which led to fewer numbers of spectra per m/z compared to that obtained by the LC-MS/MS method. Therefore, in this report, the number of unique peptides identified for a given protein was used to indicate the relative protein abundance for proteins identified by the LC-MALDI MudPIT method. In total, protein abundances of 584 proteins identified in two replicates by Mascot or Protein Pilot were estimated and compared to their relative mRNA levels. The calculated correlation coefficient for the LC-MALDI MudPIT method is 0.3 (**Figure 4-3C**). Meanwhile, proteins identified by Mascot or Protein Pilot in one or two replicates (n = 1506) by the LC-MALDI MudPIT method were also used to calculate the correlation coefficient, and the r is 0.22.

As a partial independent validation of the quantification method we used for proteins identified by the LC-MALDI MudPIT method, proteins which were identified in both gel-based and LC-MALDI MudPIT method were further analyzed. We reasoned that since gel-based methods are well-known to preferentially identify abundant proteins (Baggerman et al., 2005), the group of proteins identified by 2D gel analysis should be of overall high abundance, as compared to the entire theoretical proteome. If our MudPIT method of protein quantification has validity, then the group of proteins identified by 2D gel analysis should be over-represented among the proteins assigned to high abundance categories from our MudPit analysis. We tested this as follows. First, the 584 proteins identified in two replicates from the MudPit analysis were divided into four categories according to their estimated protein abundance (146 proteins in each category): a) highly abundant, b) abundant, c) moderate and d) low. Compared to the BR method, 14% (21 proteins), 8% (12 proteins), 3% (four proteins), and 0.7% (one protein) from category a, b, c, and d were also identified by the BR method, respectively. Compared to the NR method, 11% (16 proteins), 3% (five proteins), 1.3% (two proteins) and 2% (three proteins) from category a, b, c, and d were also identified by the NR method, respectively. Statistical analysis showed that P values were 8.8E-9 and 5.3E-9 for comparison datasets between BR and LC-MALDI MudPIT, and between NR and LC-MALDI MudPIT. These results strongly suggest that proteins assigned to the high

abundance category in analysis of our LC-MALDI MudPIT data are in fact high abundance proteins, and support our method of protein abundance evaluation for the MudPIT dataset.

**Factors correlated to the correlation between mRNA and protein levels in guard cells**

In total, 584 proteins identified with Protein Pilot or Mascot algorithms in two independent MudPIT samples were used to calculate the correlation coefficient. These 584 proteins were further classified into different gene ontology (GO) categories using GO software. GO categories from these 584 proteins for Cellular Component, Biology Process and Molecular Function containing more than 100 of the proteins identified by our MudPIT analysis in two replicates were used for further analysis. The abundance each of protein in each of these GO categories was the compared to its corresponding mRNA level (**Table 4-3**). The correlation coefficient varied from 0.23 to 0.62 and the highest correlation coefficient was found for proteins in the Cellular Component GO category "protein metabolism" ($r = 0.62$) (**Table 4-3**).

**Co-regulation between mRNA and protein levels with ABA treatment or mutation of *GPA1***

For technical reasons, microarray technology is more accurate for comparison of changes in levels of a transcript than for absolute transcript quantification, and this fact may contribute to poor correlation coefficients in microarray transcriptome vs. proteome comparisons. We were also interested in assessing how manipulations previously shown to strongly regulate stomatal function, and in particular ABA signaling (Schroeder et al., 2001a; Fan et al., 2004; Li et al., 2006), would result in co-regulation of the guard cell transcriptome and proteome. Accordingly, correlations between mRNA and protein

abundances upon ABA treatment or null mutation of *GPA1* were also examined in guard cells. We previously performed iTRAQ proteomic analyses to assess changes in protein abundance in wild-type and *gpa1* mutant guard cells, with or without ABA treatment (Chapter 3). After imposition of highly stringent selection criteria (proteins were identified in two or three replicates, and; 2) had iTRAQ ratios were higher than 1.17 or lower than 0.85, and; 3) P values of the iTRAQ ratios were less than 0.05), we previously showed that two and six proteins had significant quantitative changes with ABA treatment in Col and *gpa1-4* guard cells, respectively, and 18 guard cell proteins were quantitatively affected by null mutation of *GPA1* (Chapter 3).

To allow us to assess whether there is a correlation between changes in transcript and protein abundances induced by genetic or experimental manipulation of ABA signaling, we obtained transcriptome data by performing, microarray experiments using highly purified guard cells isolated from Col and *gpa1-4* plants with or without ABA treatment. Transcripts encoding three proteins present in our iTRAQ dataset, rbcs2B, At1g67090 and TGG2, were not represented by probes on the microarray chip (**Table 4-4**), and thus were not considered further. Twenty-one genes were not significantly affected at the transcriptional level (**Table 4-4**). Microarray results showed that only two genes, *MDH* and *lhcb6*, showed altered abundance at the transcriptional level, exhibiting downregulation in response to ABA in Col and *gpa1-4* guard cells, respectively (**Table 4-4**). Compared to the proteomic data, MDH exhibits opposite changes at the transcriptional (down-regulated) and translational levels (up-regulated), and lhcb6 was quantitatively down-regulated at both mRNA and protein levels. These results suggest that the co-regulation of mRNA and protein levels was poor with ABA treatment or mutation of *GPA1* in Arabidopsis guard cells; however, this conclusion is tempered by the small sample size (n = 26). When the dataset is expanded to include all proteins identified in at least two of the three independent biological samples without considering P values and fold change cutoff, similarly poor co-regulation results were obtained (**Figure 4-4**).

## Discussion

### Comparison of transcriptome and proteome correlations in Arabidopsis guard cells vs. other systems.

Recently, modest correlation between mRNA and protein abundances in six Arabidopsis organs was detected. The highest correlation coefficient, calculated with at least 4,800 proteins each, was from leaves (0.68), followed by flowers (0.59), roots (0.59), siliques (0.57), cell culture (0.57) and seeds (0.52) (Baerenfaller et al., 2008). In this study, a total of ~13,000 proteins from these six Arabidopsis organs was identified by LTQ (Linear Trap Quadrupole) ion trap mass spectrometry (Baerenfaller et al., 2008). Microarray data obtained from "organs that most closely resembled those organs included in the proteome analysis" (Baerenfaller et al., 2008) were extracted from Genevestigator to assess the correlation between the transcriptome and the proteome in these organs (Baerenfaller et al., 2008). Similar positive correlation was also detected in the budding yeast, *Saccharomyces cerevisiae*, by several groups (Gygi et al., 1999a; Ideker et al., 2001; Griffin et al., 2002; Washburn et al., 2003). A comparison of transcriptomic and proteomic results on yeast from those groups was reviewed by Hack, and the range of correlation coefficients was 0.46-0.76 (Hack, 2004). In a combination of published yeast proteomic and transcriptomic datasets, 2,000 proteins in total (~40% of the proteome) were used to calculate the global correlation coefficient between mRNA and protein abundance in yeast ($r = 0.66$) (Greenbaum et al., 2003). These results suggest that proteins are mainly regulated at the transcriptional level at the genomic scale in Arabidopsis and yeast.

The correlation coefficients using proteins identified in two replicates by BR, NR and LC-MALDI MudPIT methods in our study were -0.06, -0.06 and 0.3, suggesting that mRNA and protein levels are not well correlated in the single cell type consisting of guard cells. Such poor correlation was also detected in human lung adecarcinomas samples. The global correlation coefficient was only -0.025 using 165 proteins identified

using 2D gels from 85 human lung adecarcinomas samples (Chen et al., 2002). However, poor correlation has not been reported for all single cell types that have been studied. Six single cell types in human prostate tissue -- endothelial, luminal, stromal, basal epithelial, stem, cancer -- were isolated by magnetic cell sorting (MACS) or laser capture microdissection (LCM) methods, and the protein abundances of 58 proteins in each single cell type were determined by an immunohistochemistry (IHC) method via specific antibody to each protein. mRNA levels corresponding to these 58 proteins were obtained by microarray methods using Affymetrix and/or Aligent arrays. It was shown in this report that the range of the Pearson correlation coefficient was -0.01 to 0.55 when cells were isolated by the MACS method and gene expression was determined using the Affymetrix array, and 0.26 to 0.57 when cells were isolated by the LCM method and gene expression was determined by Affymetrix and Aligent arrays (Pascal et al., 2008). These data lead us to conclude that correlation between mRNA and protein levels may differ in different cell types. Protein quantification via IHC method is assessing protein abundance via binding ability of antibodies to proteins and protein extraction is not required (Netea-Maier et al., 2008), which is why this may be a relatively more accurate method for determination of protein abundance compared to 2D gel and LC-MS/MS methods. This might be one reason why the calculated correlation coefficient in our study is relatively lower compared to the study by Pascal et al. (2008).

**Technological reasons affect the calculated correlation in Arabidopsis guard cells**

The poor correlation between transcriptome and proteome observed for guard cells may be caused by technological limitations. It has been suggested that one major reason for the low correlation between mRNA and protein levels in *D. vulgaris* was the inaccuracy in estimation of protein abundance by the LC-MS/MS proteomic method, and the correlation was increased when only reproducible proteomic data (high reproducibility between replicates) were used (Nie et al., 2006). In our study, when proteins identified in two LC-MALDI MudPIT samples (defined as the reproducible data

for our study) by Mascot or Protein Pilot were used, the correlation coefficient was 0.3 (**Figure 4-3 C**) and the correlation coefficient decreased to 0.22 if proteins identified in one LC-MALDI MudPIT replicate by Mascot or Protein Pilot were also included (**Figure 4-3 D**). These results suggest that the correlation was increased when more reliable data, namely proteins identified in two replicates were used. This notion was supported by further analysis. For proteins which were identified by both 2D gel and LC-MALDI MudPIT methods in two replicates, we further compared the correlation coefficients using protein amounts quantified in different methods by the gel-based vs. gel-free methods. For the 38 proteins identified by both BR and LC-MALDI MudPIT methods, the correlation coefficients were -0.08 vs 0.20 using protein amounts estimated for BR and LC-MALDI MudPIT; while for the 26 proteins identified in both NR and LC-MALDI MudPIT methods, the correlation coefficients were 0.08 vs -0.02 using protein amounts estimated for NR and LC-MALDI MudPIT. These data indicate that the protein quantification method can significantly affect the calculated correlation between mRNA and protein abundance.

**Biological reasons affect the correlation in Arabidopsis guard cells**

There is a general agreement that proteins with highly abundant transcripts are more likely to be present in the identified proteome (**Table 4-2**), and that proteins with low abundance are more likely to be absent from the transcriptome (**Figure 4-2**). However, when all proteins identified in two replicates are considered, the calculated correlation coefficient between the transcriptome and the proteome is low (**Figure 4-3**). The poor correlation between mRNA and protein levels in guard cells may due to biological reasons, such as RNA and protein turnover rates, protein function and stability, and post-translational modifications (Hegde et al., 2003). In *D. vulgaris,* it was reported that the correlation between mRNA and protein abundance was affected by the functional category of the given genes/proteins (Nie et al., 2006), and in *S. cerevisiae*, the correlation improved when genes/proteins involved in certain protein pathways were used

(Washburn et al., 2003). In our case, the highest correlation (r = 0.62) was detected for proteins in the GO category "protein metabolism", suggesting that transcriptional regulation is an important regulatory mechanism in the protein metabolism process in guard cells.

It is well know that guard cells, a single cell type which, unlike most plant cell types, lack plasmodesmatal connections to neighboring cells, respond independently to numerous environmental cues, e. g drought, humidity, $CO_2$, light intensity, and pathogens (Fan et al., 2004), and multiple plant hormones, e. g. ABA, ethylene and auxin (Assmann, 1993; Blatt, 2000; Assmann and Wang, 2001; Israelsson et al., 2006; Pandey et al., 2007; Shimazaki et al., 2007; Fan et al., 2008). The poor correlation between mRNA and protein abundance may partly result from the highly diverse functions of guard cells. This is consistent with the fact that when subsets of proteins with different functions or subcellular localizations were used (**Table 4-3**), five out of the seven (71%) calculated correlation coefficients were greater than the overall value of 0.3. Another possible biological reason for the poor correlation is the inherent nature of the function which guard cells have evolved to execute, namely rapid responses and volume changes in response to stimuli (Ng et al., 2004; Shope and Mott, 2006; Young et al., 2006). Guard cells respond to stimuli with such rapidity (on the order of seconds, in some cases) that modification in signal transduction is likely to be initiated at steps downstream of de novo transcription. Our iTRAQ data further suggested that post-translational modification rather than changes in protein abundance might be the main regulatory mechanism upon ABA treatment in guard cells (Chapter 3).

Poor coregulation between mRNA and protein abundance upon ABA treatment or *GPA1* mutation in guard cells was detected. One technological explanation for the poor co-regulation upon ABA treatment or *GPA1* mutation is that proteomic methods can only detect relatively abundant proteins, and most of the proteins which have changes at the transcriptional levels cannot be detected by proteomic method (Dihal et al., 2008). The poor coregulation between mRNA and protein levels upon ABA treatment or *GPA1* mutation in Arabidopsis guard cells may also due to the poor correlation between mRNA and protein levels in Arabidopsis guard cells. Biologically, for the small set of 26 proteins

identified with significantly altered abundance by the iTRAQ study (Chapter 3), regulation at protein level in GPA1- and/or ABA-regulated signal pathways might be the main regulatory mechanism in guard cells.

**Protein abundance does not affect correlation in Arabidopsis guard cells**

In yeast, stronger correlations between mRNA and protein levels were found for proteins identified from 2D gels than for proteins identified from shotgun methods, leading to the suggestion that abundant proteins tend to be better correlated with their mRNA levels in yeast (Hack, 2004). However, this hypothesis is not supported by our data. Compared to the correlation coefficient obtained using proteins identified in two replicates from the LC-MALDI MudPIT method (r= 0.3), the correlation coefficients between the transcriptome and the proteome in guard cells were lower (r = -0.06 for BR and NR) when proteins identified from the 2D gels were used. Since abundant proteins are most likely to be detected by gel-based methods, our results suggest that the correlation coefficient between the transcriptome and the proteome in guard cells is not improved by considering only the subset of relatively abundant proteins.

In summary, poor correlation between mRNA and protein levels was observed in Arabidopsis guard cells, which might contribute to the poor co-regulation between mRNA and protein levels upon ABA treatment or *GPA1* mutation. Both biological and technological reasons likely contribute to the low correlation between mRNA and protein abundance in guard cells, and the extent of correlation differs for proteins of different subcellular localizations and different functional categories.

## Materials and Methods

### Protein quantification

Broad range and narrow range pH 2D gels were stained with Coomassie Brilliant Blue (CBB) G250 and Sypro-Ruby respectively (Chapter 2). A master gel was created *in silico* using PDQuest software for BR and NR 2D gels using two independent gels (total of four gels). All spots simultaneously identified by two replicates were represented on the master gel. The absolute protein amount in each protein spot corresponding to a spot on the master gel was quantified according to the spot intensity by PDQuest software (Bio-Rad). All protein spots known to contain multiple proteins were eliminated from the analysis. The relative abundances of the proteins identified by the MudPIT method using either the Mascot or Protein Pilot search algorithm were estimated as the number of unique peptides identified for each protein.

### Guard cell preparation for microarray experiments (text provided by Dr. Sona Pandey)

About 600 healthy leaves from five week old plants were cut and divided into two batches. Each batch of leaves was blended for 60 seconds and filtered through a 100 μm mesh (The Spectrum Companies) to remove broken mesophyll and epidermal cells. Blended peels from each sample were washed with water until all the foam was gone and then transferred to 300 mL enzyme solution. Peels were then digested for 75 minutes at 25-27$^{o}$C in a shaking water bath at 140 excursions per minute. The digested peels were finally filtered through 30 μm mesh and washed with 500 mL basic solution. The washed peels were further used for RNA isolation. Enzyme solution was prepared by adding 0.7% cellulysin, 0.1% PVP40, 0.25% BSA, 0.5 mM ascorbic acid in 55% basic solution

plus 45% DD water. Basic solution contained 5 mM MES-TRIS, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM ascorbic acid, 10 μM $KH_2PO_4$, pH 5.5.

**RNA isolation (Performed and text provided by Dr. Sona Pandey)**

RNA was isolated using Trizol (Invitrogen, Carsbad, CA) according to the manufacturer's protocol with the following modifications. After adding isopropanol, the RNA was precipitated at -20$^{o}$C for one hour, followed by a spin at 4$^{o}$C for 30 minutes to recover the precipitate. High salt solution (HSS; recommended for plant tissues by the manufacturer) was also added during precipitation. (250 μL of Isopropanol and 250 μL of HSS were added per mL of TriZol reagent). RNA was finally resuspended in 20 μL in each tube and pooled into a single tube. 10 μL of DEPC treated water was added to the tubes to resuspend any remaining RNA left in the tubes. Final RNA volume was adjusted to 100 μL and was stored frozen at -80$^{o}$C for further use.

**cDNA and cRNA synthesis (Performed and text provided by Dr. Sona Pandey)**

cRNA was synthesized following a prescribed protocol (Affymetrix expression technical manual 701024 Rev.3) with slight modification. Single and double stranded cDNA was synthesized using a one-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA) with 8 μl of RNA solution (2-8 μg total RNA). For each cDNA synthesis, poly-A spike RNA (proportional to the amount of initial RNA) was added to the solution. After synthesis of single stranded cDNA, double stranded cDNA was produced following the protocol. The double stranded cDNA was purified using Sample Cleanup Module (Affymetrix, Santa Clara, CA). Twelve μL of double-stranded cDNA solution was used to synthesize cRNA with an extra 1,000 units (5 μL) of T7 RNA polymerase (Ambion, Austin, TX) added. cRNA synthesis was carried out at 37ºC for 16 hours using GeneChip IVT labeling kit (Affymetrix, Santa Clara, CA). cRNA was purified using Sample

Cleanup Module (Affymetrix, Santa Clara, CA). The amount of synthesized cRNA level was determined by subtracting the original RNA amount. Eight to 20 μg of cRNA was fragmented to 35-200 nts according to the protocol provided by Affymetrix, and used for hybridization. The transcripts were labeled and hybridized at the Pennsylvania State University, microarray core facility. Three independent experiments were performed.

**Hybridization and microarray data analysis (Performed and text provided by Song Li)**

Microarray data preprocessing and normalization were carried out using R (http://www.r-project.org), and "affy" packages (version 1.18.2) (Irizarry et al., 2003) from the Bioconductor project (http://www.bioconductor.org). Differentially expressed genes were identified using moderate-t-test implemented in Limma package (version 2.14.5) (Smyth, 2004).

## References

**Assmann, S.M.** (1993). Signal transduction in guard cells. Annu Rev Cell Biol **9,** 345-375.

**Assmann, S.M., and Wang, X.Q.** (2001). From milliseconds to millions of years: guard cells and environmental responses. Curr Opin Plant Biol **4,** 421-428.

**Baerenfaller, K., Grossmann, J., Grobei, M.A., Hull, R., Hirsch-Hoffmann, M., Yalovsky, S., Zimmermann, P., Grossniklaus, U., Gruissem, W., and Baginsky, S.** (2008). Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science **320,** 938-941.

**Baggerman, G., Vierstraete, E., De Loof, A., and Schoofs, L.** (2005). Gel-based versus gel-free proteomics: a review. Comb Chem High Throughput Screen **8,** 669-677.

**Barski, A., Cuddapah, S., Cui, K., Roh, T.Y., Schones, D.E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K.** (2007). High-resolution profiling of histone methylations in the human genome. Cell **129,** 823-837.

**Berkelman, T.** (2008). Quantitation of protein in samples prepared for 2-D electrophoresis. Methods Mol Biol **424,** 43-49.

**Blatt, M.R.** (2000). $Ca^{2+}$ signalling and control of guard-cell volume in stomatal movements. Curr Opin Plant Biol **3,** 196-204.

**Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D.H., Johnson, D., Luo, S., McCurdy, S., Foy, M., Ewan, M., Roth, R., George, D., Eletr, S., Albrecht, G., Vermaas, E., Williams, S.R., Moon, K., Burcham, T., Pallas, M., DuBridge, R.B., Kirchner, J., Fearon, K., Mao, J., and Corcoran, K.** (2000). Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. Nat Biotechnol **18,** 630-634.

**Chen, G., Gharib, T.G., Huang, C.C., Taylor, J.M., Misek, D.E., Kardia, S.L., Giordano, T.J., Iannettoni, M.D., Orringer, M.B., Hanash, S.M., and Beer, D.G.** (2002). Discordant protein and mRNA expression in lung adenocarcinomas. Mol Cell Proteomics **1,** 304-313.

**Corbin, R.W., Paliy, O., Yang, F., Shabanowitz, J., Platt, M., Lyons, C.E., Jr., Root, K., McAuliffe, J., Jordan, M.I., Kustu, S., Soupene, E., and Hunt, D.F.** (2003). Toward a protein profile of *Escherichia coli*: comparison to its transcription profile. Proc Natl Acad Sci U S A **100,** 9232-9237.

**Fan, L.M., Zhao, Z., and Assmann, S.M.** (2004). Guard cells: a dynamic signaling model. Curr Opin Plant Biol **7,** 537-546.

**Fan, L.M., Zhang, W., Chen, J.G., Taylor, J.P., Jones, A.M., and Assmann, S.M.** (2008). Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ- and RGS-deficient Arabidopsis lines. Proc Natl Acad Sci U S A **105,** 8476-8481.

**Greenbaum, D., Colangelo, C., Williams, K., and Gerstein, M.** (2003). Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol **4,** 117.

**Griffin, T.J., Gygi, S.P., Ideker, T., Rist, B., Eng, J., Hood, L., and Aebersold, R.** (2002). Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. Mol Cell Proteomics **1,** 323-333.

**Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R.** (1999a). Correlation between protein and mRNA abundance in yeast. Mol Cell Biol **19,** 1720-1730.

**Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., and Aebersold, R.** (1999b). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol **17,** 994-999.

**Hack, C.J.** (2004). Integrated transcriptome and proteome data: the challenges ahead. Brief Funct Genomic Proteomic **3,** 212-219.

**Hegde, P.S., White, I.R., and Debouck, C.** (2003). Interplay of transcriptomics and proteomics. Curr Opin Biotechnol **14,** 647-651.

**Ideker, T., Thorsson, V., Ranish, J.A., Christmas, R., Buhler, J., Eng, J.K., Bumgarner, R., Goodlett, D.R., Aebersold, R., and Hood, L.** (2001). Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science **292,** 929-934.

**Immink, R.G.H., Ferrario, S., Busscher-Lange, J., Kooiker, M., Busscher, M., and Angenent, G.C.** (2003). Analysis of the petunia MADS-box transcription factor family. Mol Genet Genomics **268,** 598-606.

**Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., and Mann, M.** (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics **4,** 1265-1272.

**Israelsson, M., Siegel, R.S., Young, J., Hashimoto, M., Iba, K., and Schroeder, J.I.** (2006). Guard cell ABA and $CO_2$ signaling network updates and $Ca^{2+}$ sensor priming hypothesis. Curr Opin Plant Biol **9,** 654-663.

**Li, S., Assmann, S.M., and Albert, R.** (2006). Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. PLoS Biol **4,** e312.

**Liu, H., Sadygov, R.G., and Yates, J.R., 3rd.** (2004). A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem **76,** 4193-4201.

**Mardis, E.R.** (2008). The impact of next-generation sequencing technology on genetics. Trends Genet **24,** 133-141.

**Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., et al.** (2005). Genome sequencing in microfabricated high-density picolitre reactors. Nature **437,** 376-380.

**Netea-Maier, R.T., Hunsucker, S.W., Hoevenaars, B.M., Helmke, S.M., Slootweg, P.J., Hermus, A.R., Haugen, B.R., and Duncan, M.W.** (2008). Discovery and validation of protein abundance differences between follicular thyroid neoplasms. Cancer Res **68,** 1572-1580.

**Ng, C.K., Kinoshita, T., Pandey, S., Shimazaki, K., and Assmann, S.M.** (2004). Abscisic acid induces rapid subnuclear reorganization in guard cells. Plant Physiol **134,** 1327-1331.

**Nie, L., Wu, G., and Zhang, W.** (2006). Correlation between mRNA and protein abundance in *Desulfovibrio vulgaris*: a multiple regression to identify sources of variations. Biochem Biophys Res Commun **339,** 603-610.

**Pandey, S., Zhang, W., and Assmann, S.M.** (2007). Roles of ion channels and transporters in guard cell signal transduction. FEBS Lett **581,** 2325-2336.

**Pascal, L.E., True, L.D., Campbell, D.S., Deutsch, E.W., Risk, M., Coleman, I.M., Eichner, L.J., Nelson, P.S., and Liu, A.Y.** (2008). Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. BMC Genomics **9,** 246.

**Reinartz, J., Bruyns, E., Lin, J.-Z., Burcham, T., Brenner, S., Bowen, B., Kramer, M., and Woychik, R.** (2002). Massively parallel signature sequencing (MPSS) as a tool for in-depth quantitative gene expression profiling in all organisms. Brief Funct Genomic Proteomic **1,** 95-104.

**Schagger, H., Cramer, W.A., and von Jagow, G.** (1994). Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal Biochem **217,** 220-230.

**Schneider, L.V., and Hall, M.P.** (2005). Stable isotope methods for high-precision proteomics. Drug Discov Today **10,** 353-363.

**Schroeder, J.I., Kwak, J.M., and Allen, G.J.** (2001a). Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature **410,** 327-330.

**Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., and Waner, D.** (2001b). Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol **52,** 627-658.

**Shimazaki, K., Doi, M., Assmann, S.M., and Kinoshita, T.** (2007). Light regulation of stomatal movement. Annu Rev Plant Biol **58,** 219-247.

**Shope, J.C., and Mott, K.A.** (2006). Membrane trafficking and osmotically induced volume changes in guard cells. J Exp Bot **57,** 4123-4131.

**Smyth, G.K.** (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol **3,** Article3.

**Tyers, M., and Mann, M.** (2003). From genomics to proteomics. Nature **422,** 193-197.

**Valor, L.M., and Grant, S.G.** (2007). Integrating synapse proteomics with transcriptional regulation. Behav Genet **37,** 18-30.

**Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W.** (1995). Serial analysis of gene expression. Science **270,** 484-487.

**Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M.** (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science **292,** 2070-2072.

**Washburn, M.P., Koller, A., Oshiro, G., Ulaszek, R.R., Plouffe, D., Deciu, C., Winzeler, E., and Yates, J.R., 3rd.** (2003). Protein pathway and complex clustering of correlated mRNA and protein expression analyses in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A **100,** 3107-3112.

**Young, J.J., Mehta, S., Israelsson, M., Godoski, J., Grill, E., and Schroeder, J.I.** (2006). $CO_2$ signaling in guard cells: calcium sensitivity response modulation, a

$Ca^{2+}$-independent phase, and $CO_2$ insensitivity of the *gca2* mutant. Proc Natl Acad Sci U S A **103,** 7506-7511.

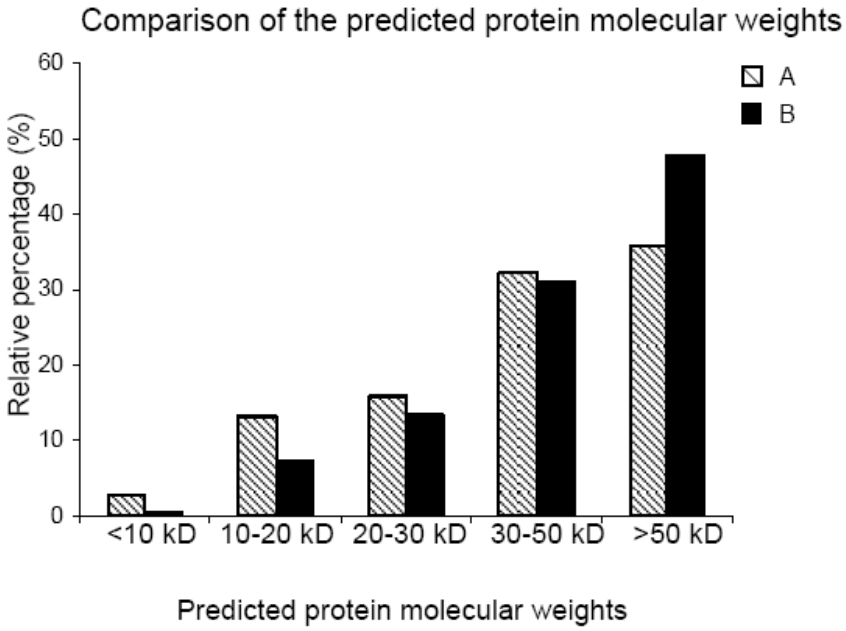



**Figure 4-1. A greater percentage of proteins have small molecular weights in the proteins whose transcripts were present in the transcriptome but were not identified by LC-MALDI MudPIT.**

The protein molecular weights were predicted by (http://www.arabidopsis.org/tools/bulk/protein/index.jsp). A. proteins whose transcripts were present in the transcriptome but were not identified by the LC-MALDI MudPIT method in one or two replicates using Mascot or Protein Pilot software. B. proteins identified by the MudPIT method in one or two replicates using Mascot or Protein Pilot software.

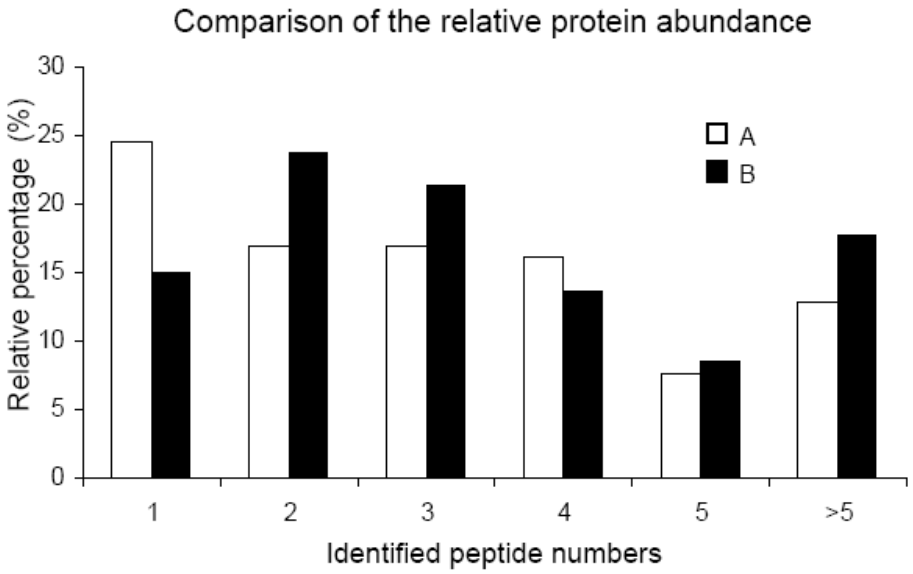



**Figure 4-2. A greater percentage of proteins whose transcripts were absent in the transcriptome but were identified by proteomic methods are low abundance proteins.**

Relative protein abundance is represented by the identified unique peptide numbers. A. proteins whose transcripts were absent from the transcriptome but were identified by the LC-MALDI MudPIT method in one or two replicates using Mascot or Protein Pilot software. B. proteins identified by the LC-MALDI MudPIT method in one or two replicates using Mascot or Protein Pilot software.

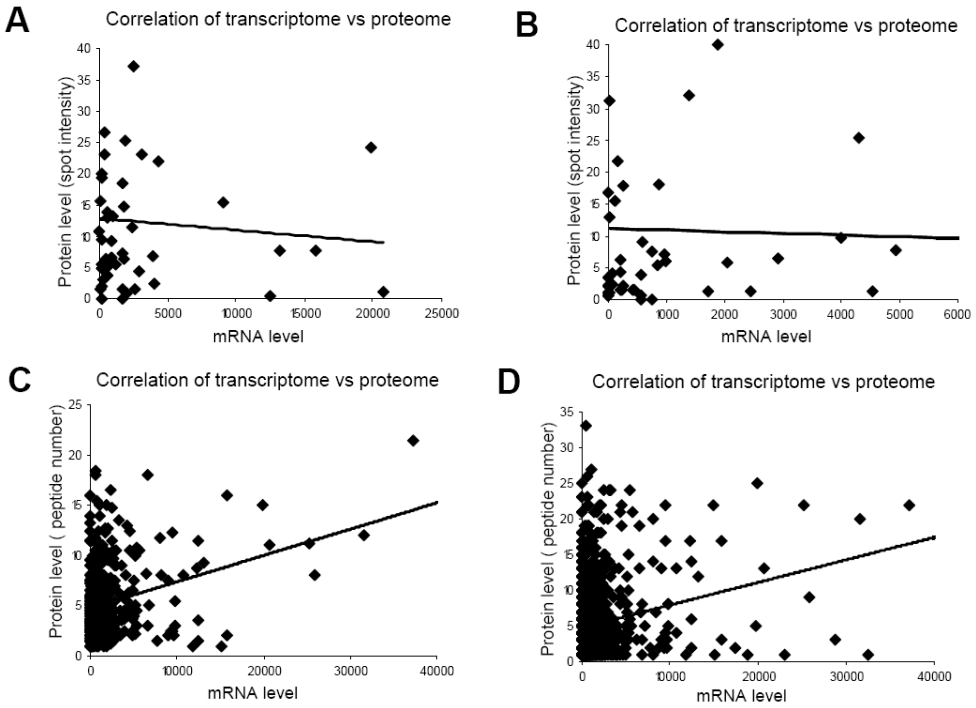



**Figure 4-3. Poor correlation between mRNA and protein levels was detected in Arabidopsis guard cells.**

The protein amounts in protein spots on BR (A, r=-0.06) and NR (B, r=-0.06) 2D gels were quantified by PDQuest software and compared to their corresponding mRNA levels. The values on y axes in Panel A and B are the spot intensity divided by $10^5$ and $10^6$ respectively. Proteins identified in two replicates from the LC-MALDI MudPIT method using Mascot or Protein Pilot software (C) were quantified by counting the number of identified unique peptides for each protein (r=0.3). D. Proteins identified by Mascot or Protein Pilot in one or two replicates (n = 1506) in the LC-MALDI MudPIT method were used to calculate the correlation coefficient (r=0.22).

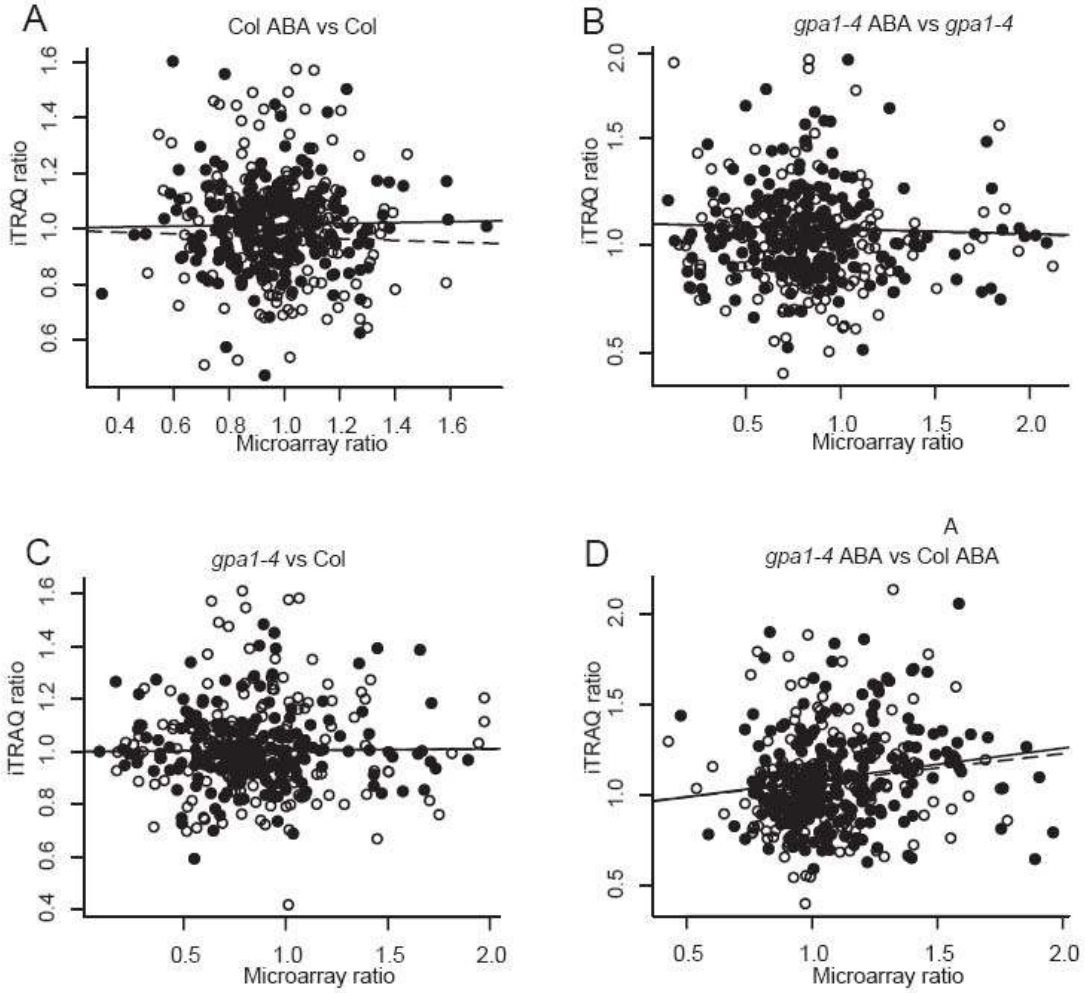



**Figure 4-4. Poor co-regulation between mRNA and protein levels was identified in Arabidopsis guard cells.**

The changes of protein abundance identified by iTRAQ study (Chapter 3) were compared to their corresponding mRNA changes. A. The comparison of protein and mRNA abundance changes with ABA treatment in Col guard cells. B. The comparison of protein and mRNA abundance changes with ABA treatment in *gpa1-4* guard cells. C. The comparison of protein and mRNA abundance changes with *GPA1* mutation in Col guard cells. D. The comparison of protein and mRNA abundance changes with ABA treatment in *gpa1-4* guard cells vs Col guard cells.
The closed circles indicate proteins identified in at least two replicates and open circles indicate proteins identified in only one replicate. The open circles plus closed circles indicate all proteins identified in the iTRAQ experiments.
The solid regression line is for closed circles and dashed regression line is for open+closed circles. In panel B and C, the open and closed circles give the same regression results, so the two lines overlapp.

**Table 4-1. 117 proteins whose transcripts were absent from the transcriptome but were identified by 2D gel and LC-MALDI MudPIT methods in one or two replicates using Mascot or Protein Pilot software.**

| AGI | Peptide # | AGI | Peptide # | AGI | Peptide # | AGI | Peptide # |
|---|---|---|---|---|---|---|---|
| At5g52320 | 1 | At1g23820 | 1 | At3g09790 | 3 | At1g31710 | 4 |
| At5g09660 | 1 | At5g22640 | 2 | At2g02540 | 3 | At1g11580 | 4 |
| At5g09300 | 1 | At1g68020 | 2 | At1g80660 | 3 | At1g08135 | 4 |
| At4g34950 | 1 | At5g60660 | 2 | At1g76400 | 3 | At2g21060 | 4 |
| At4g32280 | 1 | At5g54190 | 2 | At1g75130 | 3 | At5g45930 | 5 |
| At4g20110 | 1 | At5g53320 | 2 | At1g73600 | 3 | At5g26280 | 5 |
| At4g18430 | 1 | At5g46570 | 2 | At1g63310 | 3 | At5g05680 | 5 |
| At4g14570 | 1 | At5g19220 | 2 | At1g56110 | 3 | At4g18670 | 5 |
| At4g13840 | 1 | At5g13980 | 2 | At1g52570 | 3 | At4g17770 | 5 |
| At4g09010 | 1 | At5g13930 | 2 | At1g23410 | 3 | At4g16590 | 5 |
| At4g08780 | 1 | At4g35300 | 2 | At1g20970 | 3 | At1g53720 | 5 |
| At3g54560 | 1 | At4g23900 | 2 | At1g10200 | 3 | At1g19370 | 5 |
| At3g52380 | 1 | At4g14680 | 2 | At3g55010 | 3 | At1g04600 | 5 |
| At3g42950 | 1 | At3g48410 | 2 | AtMg00510 | 4 | At3g45140 | 6 |
| At3g24480 | 1 | At2g30200 | 2 | At5g55040 | 4 | At5g59370 | 6 |
| At3g18890 | 1 | At2g22230 | 2 | At5g49555 | 4 | At3g01670 | 7 |
| At3g14570 | 1 | At2g18450 | 2 | At5g07460 | 4 | At5g55660 | 7 |
| At2g26730 | 1 | At1g75040 | 2 | At2g39850 | 4 | At2g46020 | 7 |
| At2g16890 | 1 | At1g65860 | 2 | At1g78370 | 4 | At1g54040 | 7 |
| At1g77060 | 1 | At1g22430 | 2 | At1g62560 | 4 | At5g14740 | 8 |
| At1g67640 | 1 | At4g05590 | 2 | At5g04440 | 4 | At2g45290 | 8 |
| At1g64370 | 1 | At5g40450 | 3 | At4g31500 | 4 | At1g75780 | 11 |
| At1g62360 | 1 | At5g23020 | 3 | At4g28650 | 4 | At3g50820 | 12 |
| At1g60810 | 1 | At3g62110 | 3 | At4g02420 | 4 | At1g10770 | 12 |
| At1g54560 | 1 | At1g73370 | 3 | At3g58990 | 4 | At5g04140 | 12 |
| At1g51410 | 1 | At5g64860 | 3 | At3g12860 | 4 | At5g57350 | 13 |
| At1g24010 | 1 | At4g30720 | 3 | At2g42710 | 4 | At1g20010 | 13 |
| At1g16820 | 1 | At3g47950 | 3 | At1g71695 | 4 | At3g12810 | 14 |
| At5g44120 | NR | At1g51490 | NR | At4g28520 | NR | At4g38950 | NR |
| At5g60720 | NR | | | | | | |

**Table 4-2. High abundance transcripts encode proteins more likely to be identified by proteomic methods.**

Proteins in B were proteins identified by BR, NR or MudPIT in one or two replicates using Mascot or Protein Pilot software.

| Abundance categories | mRNA level (log2) | A Total number of unique transcripts | B Transcripts for which corresponding proteins were identified. | (B/A)x100 |
|---|---|---|---|---|
| 1 | 2-4 | 8644 | 73 | 0.8 |
| 2 | 4-6 | 2637 | 58 | 2.2 |
| 3 | 6-8 | 1625 | 88 | 5.4 |
| 4 | 8-10 | 2336 | 165 | 7.1 |
| 5 | 10-12 | 2713 | 303 | 11.2 |
| 6 | >12 | 4856 | 884 | 18.2 |

**Table 4-3. Transcriptome/proteome correlation coefficients are correlated with protein functions and subcellular localizations.**

| **GO category-GO Cellular component** | **Pearson correlation coefficient (r)** |
|---|---|
| Chloroplast | 0.28 |
| Plastid | 0.23 |
| Mitochondria | 0.47 |
| Plasma membrane | 0.45 |
| **GO category-GO Biological process** | **Pearson correlation coefficient (r)** |
| Response to abiotic and biotic stimulus | 0.43 |
| Response to stress | 0.41 |
| Protein metabolism | 0.62 |

* Only GO categories containing more than 100 proteins of the proteins identified by MudPIT in two replicates using Mascot or Protein Pilot software were evaluated.

**Table 4-4. Poor co-regulation of mRNA and protein abundance in Arabidopsis guard cells upon treatment with ABA or by null mutation of *GPA1*.**

Three biological replicates were performed for iTRAQ and microarray experiments.

| AGI | Protein name | iTRAQ comparison | Average Protein fold change | Average mRNA fold change | Microarray P value |
|---|---|---|---|---|---|
| At1g53240 | MDH | ColABA vs Col | 1.36 | 0.32 | 0.002 |
| At5g38420 | rbcS2B | ColABA vs Col | 1.18 | NA | NA |
| At1g15820 | lhcb6/CP24 | *gpa1-4*ABA vs *gpa1-4* | 0.74 | 0.49 | 0.002 |
| At1g76180 | ERD14 | *gpa1-4*ABA vs *gpa1-4* | 0.75 | 1.47 | 0.31 |
| AtCg00280 | PsbC | *gpa1-4*ABA vs *gpa1-4* | 1.58 | 0.79 | 0.63 |
| AtCg00340 | PsaB | *gpa1-4*ABA vs *gpa1-4* | 1.483 | 0.89 | 0.85 |
| AtCg00350 | PsaA | *gpa1-4*ABA vs *gpa1-4* | 1.45 | 0.94 | 0.96 |
| AtCg00680 | PsbB | *gpa1-4*ABA vs *gpa1-4* | 1.39 | 0.95 | 0.96 |
| At1g06680 | PsbP1 | *gpa1-4* vs Col | 1.34 | 0.82 | 0.97 |
| At1g20340 | PETE2/ DRT112 | *gpa1-4* vs Col | 2.02 | 0.73 | 0.88 |
| At1g20620 | CAT3 | *gpa1-4* vs Col | 1.42 | 1.43 | 0.91 |
| At1g42970 | GAPB | *gpa1-4* vs Col | 1.23 | 0.62 | 0.95 |
| At1g67090 | Rubisco small subunit | *gpa1-4* vs Col | 1.82 | NA | NA |
| At1g76180 | ERD14 | *gpa1-4* vs Col | 2.14 | 0.97 | 1 |
| At1g79040 | PsbR | *gpa1-4* vs Col | 1.54 | 0.76 | 0.62 |
| At2g39730 | RAC | *gpa1-4* vs Col | 1.95 | 0.99 | 1 |
| At3g55800 | SBPase | *gpa1-4* vs Col | 1.46 | 0.62 | 0.82 |
| At3g60750 | transketolase-like protein | *gpa1-4* vs Col | 1.45 | 0.99 | 1 |
| At3g62030 | ROC4 (CYP20-3) | *gpa1-4* vs Col | 1.47 | 0.73 | 0.97 |
| At5g25980 | TGG2 | *gpa1-4* vs Col | 1.54 | NA | NA |
| At5g55660 | Unknown protein | *gpa1-4* vs Col | 0.65 | 0.68 | 0.87 |
| At5g60640 | PDIL1-4 | *gpa1-4* vs Col | 1.42 | 0.87 | 0.98 |
| At5g66570 | PSBO1 | *gpa1-4* vs Col | 1.67 | 0.75 | 0.95 |
| AtCg00120 | ATP synthase CF1 alpha subunit | *gpa1-4* vs Col | 1.43 | 0.96 | 1 |
| AtCg00480 | ATPB | *gpa1-4 vs* Col | 1.35 | 1.19 | 0.97 |
| AtCg00490 | RBCL | *gpa1-4 vs* Col | 1.44 | 0.92 | 1 |

NA: Not available.

# Chapter 5

## Identification of GPA1 interacting proteins

## Introduction

Heterotrimeric G proteins are major components of signal transduction pathways in both mammals and plants. There are multiple α, β, and γ subunits (about 23 Gα, 5 Gβ and 12 Gγ; >1380 combinations) in mammals (Assmann, 2004; McCudden et al., 2005), which contributes to the functional diversity of G proteins in mammals. By contrast, although G proteins have been implicated in multiple signaling pathways, only one Gα (*GPA1*), one Gβ (*AGB1*) and two Gγ (*AGG1* and *AGG2*) subunits have been identified in Arabidopsis (Assmann, 2002, 2004; Temple and Jones, 2007). Formation of the Gαβ heterodimer (Fan et al., 2008) and the Gαβγ heterotrimer (Wang et al., 2008) have been reported in Arabidopsis, indicating that similar molecular mechanisms of G protein signaling may exist in mammalian and plant systems. Meanwhile, GPA1 was also detected in large plasma membrane complexes (~ 700 kD) in Arabidopsis (Wang et al., 2008), indicating GPA1 may interact with multiple effectors to induce downstream signals in Arabidopsis. To date, except Gβ and Gγ subunits, seven proteins have been detected to interact with GPA1 (**Table 1-2**), including two G protein coupled receptors, GCR1 (G protein-coupled receptor) (Pandey and Assmann, 2004) and GCR2 (Liu et al., 2007), and five effectors, AtRGS1 (regulator of G protein signaling) (Chen et al., 2003), PLDα1 (phospholipase Dα1) (Zhao and Wang, 2004), PD1 (prephenate dehydratase) (Warpeha et al., 2006), AtPirin1 (Lapik and Kaufman, 2003) and THF1 (thylakoid formation) (Huang et al., 2006). A detailed introduction to G protein functions has been described in Chapter 1. Here I focus on techniques I used for discovering GPA1 interacting proteins and preliminary results obtained using these technologies.

Multi-protein complex formation can occur extracellularly and intracellularly and is essential for many cellular processes. Yeast two hybrid screening is a molecular biology technique to assess pairs of candidate interactors or discover new interacting partners for known proteins by screening a candidate library (Young, 1998). It is based on reconstitution of transcription factor activity and must occur in the nucleus, which

makes it unsuitable for identification of membrane protein interactions (Iyer et al., 2005). The Split Ubiquitin System (SUS), known as a yeast two hybrid system for membrane proteins, was first introduced by Johnsson and Varshavsky in 1994 to test protein-protein interactions and is especially good for evaluating interactions between membrane proteins (Johnsson and Varshavsky, 1994; Pandey and Assmann, 2004). Ubiquitin (Ub) is a small and highly conserved protein and its C-terminal tagged proteins can be recognized and cleaved by ubiquitin-specific proteases (UBPs). A native ubiquitin can be split into two halves: an N-terminal (Nub) and a C-terminal (Cub) half. These two parts have affinities to each other and can spontaneously reform a native ubiquitin (Johnsson and Varshavsky, 1994). However, modification of the Nub part (NubG) disrupts this spontaneous interaction. If two potentially interacting proteins are fused separately to mutant NubG and Cub parts, the interaction will bring NubG and Cub parts close enough to activate UBP. The artificial transcriptional factor PLV will be excised from the C-terminal of the Cub vector by the activated UBP. Once PLV enters the nucleus, it will induce expression of the reporter genes. Expression of the reporter genes can be used to select yeast colonies which host the interacting proteins (Johnsson and Varshavsky, 1994). Since the Cub vector utilizes a Met-repressible *MET25* promoter, high concentration of Met (1 mM) can significantly decrease nonspecific interactions (Obrdlik et al., 2004). GCR1 (Pandey and Assmann, 2004), RGS1 (Chen et al., 2003) and AGB1 (Chen et al., 2006) were successfully shown to be GPA1 interacting proteins by the SUS method (see Chapter 1 for details).

GPA1 is localized at the plasma membrane in Arabidopsis (Adjobo-Hermans et al., 2006; Wang et al., 2007). On the basis of the G protein signaling scenario in mammalian cells, I chose several candidate G protein effectors for evaluation in Arabidopsis. $K^+$ channels, phosphatidylinositol-3-kinase (PI3K), phospholipase C (PLC) and phospholipase D (PLD) (Jones and Assmann, 2004). Fifteen genes encoding $K^+$ channels have been identified in the Arabidopsis genome (Very and Sentenac, 2002). In the Arabidopsis genome, there are nine genes encoding PLCs (Hunt et al., 2004), 12 genes encoding PLDs (Qin and Wang, 2002) and only one PI3K gene (Welters et al., 1994) (**Table 5-1**). PI3K, $K^+$ channels, PLCs and PLDs have been shown to be involved

in ABA signaling in guard cells either in faba bean or Arabidopsis (Wang et al., 2001; Mueller-Roeber and Pical, 2002; Park et al., 2003), importantly, it was demonstrated that PLDα1 could interact with GPA1 (Zhao and Wang, 2004) and lack of this interaction could increase the stomatal sensitivity to ABA in inhibition of stomatal opening (Mishra et al., 2006). I therefore evaluated the interaction of these proteins with GPA1 via SUS assay.

Besides SUS, I also used three other methods to discover GPA1 interaction partners, including tandem affinity purification (TAP), co-immunoprecipitation and blue native 2D gel. TAP technology was first developed for rapid purification of protein complexes from yeast (Rigaut et al., 1999). TAP has two IgG-binding domains of protein A of *Staphylococcus aureus* (ProtA) and a calmodulin binding peptide (CBP) linked by a tobacco etch virus (TEV) protease cleavage site (Rigaut et al., 1999). The TAP tag can be fused to either the N- or the C- terminus of the target protein, and the construct encoding the fusion protein can be introduced into a host cell or organism. Expressed TAP-tagged protein along with its interacting partners is sequentially affinity-purified by its two tags (Puig et al., 2001; Bauer and Kuster, 2003). Although the TAP method was first used in yeast, it was successfully adapted to Arabidopsis recently (Witte et al., 2004; Rubio et al., 2005). Protein complex purification by the TAP method can dramatically reduce co-purification of non-specific binding proteins. Combined with mass spectrometry (MS), the TAP method is also suitable for large-scale protein complex identification (Bauer and Kuster, 2003). One limitation is associated with the relative large TAP tag. The original TAP tag encodes 181 amino acids and might interfere with protein modifications and protein-protein interactions (Feng et al., 2004). This limitation can be minimized using modified TAP tags containing relatively smaller FLAG (8 amino acids) and HA (9 amino acids) tags. In order to enhance the TAP efficiency, 3 FLAG and 3 HA tags were used in tandem in our study.

Co-immunoprecipitaiton combined with MS is a recently developed powerful tool to study the protein interactome *in vivo*. During the coimmunoprecipitation procedure, an antibody to the protein of interest is used to form the antibody-protein complex. Then the complex is precipitated using protein-G or protein-A sepharose which can bind to the

antibody. Any protein complexed with the targeted protein can be co-precipitated and confirmed by western blot, if an antibody exists, or identified via MS sequencing (Berggard et al., 2007). In our study, the GPA1 antibody was used to co-immunoprecipitate GPA1 interacting partners.

The blue native/SDS 2D gel electrophoresis technique was first developed to analyze mitochondrial membrane protein complexes (Schagger and von Jagow, 1991), and later on it was applied to investigate native functional protein complexes (Reisinger and Eichacker, 2007). In native 2D gel electrophoresis, non-denatured proteins are first separated in a native PAGE gel, followed by separation in a second dimension SDS-PAGE gel. Protein-protein interactions are preserved in the first dimension. Once denatured, the protein complexes are separated by protein molecular mass in the second dimension. In theory, proteins in the same vertical line on the second dimension may form protein complexes (Schagger and von Jagow, 1991). Native 2D has been widely used to analyze membrane protein complexes (Lis et al., 2003; Camacho-Carvajal et al., 2004).

In this chapter, I describe preliminary results from SUS, CoIP, TAP and blue native 2D gel electrophoresis methods in identification of GPA1 interacting proteins. These studies provide useful information for further investigation of GPA1 interacting proteins. Several candidates were found by SUS, including GPA1, PLC1, PLC2 and PLDβ1, and confirmation of the interactions was also pursued.

## Results

### GPA1 interacting candidates identified by SUS

Interactions of 18 candidate proteins with GPA1 were tested by the SUS method (**Table 5-1**) (see Material and methods for SUS procedure). At least three independent replicates were performed to test the interaction between GPA1 and each candidate

GPA1 interacting protein. On the basis of the yeast growth assay (**Figure 5-1**, **Table 5-2**), it was shown that the GPA1-Cub could interact with all the C-terminal Nub-G tagged proteins on plates without Met supplement, but did not interact with any proteins on either 200 μM or 1 mM Met plates (**Figure 5-1A**); the GPA1-Cub could interact with all the N-terminal Nub-G tagged proteins on plates without Met supplement, but only interacted with NubG-GPA1 (**Figure 5-1B**, 12), NubG-PLC2 (**Figure 5-1B**, 15) and NubG-PLDβ1 (**Figure 5-1B**, 19) on either 200 μM or 1 mM Met plates. NubG-PLC1 (**Figure 5-1B**, 14) showed weak interactions with GPA1-Cub on plates with supplement of either 200 μM or 1 mM Met (**Figure 5-1B**). GPA1-NubG could interact with Cub-tagged GPA1, PI3K, PLC1, PLC2, PLDα4, PLDβ1 and PLDδ1 on plates without Met, but only interacted with PI3K-Cub, PLC1-Cub, PLC2-Cub and PLDβ1-Cub on plates with supplement of 200 μM and 1 mM Met (**Figure 5-1C**). Similar to GPA1-NubG, NubG-GPA1 could interact with Cub-tagged GPA1, PI3K, PLC1, PLC2, PLDα4, PLDβ1 and PLDδ1 on plates without Met, but only interacted with GPA1-Cub, PI3K-Cub, PLC1-Cub, PLC2-Cub, PLDβ1-Cub and PLDδ1-Cub on plates with 200 μM and 1 mM Met (**Figure 5-1D**). In summary, on the basis of the yeast growth assay on 1 mM Met, NubG-GPA1, PLC2 and PLDβ1 were found to interact with GPA1 tagged by Cub. Reciprocally, PLC1, PLC2 and PLDβ1-Cub were detected to interact with GPA1 tagged by NubG at either N- or C-terminus. PLDδ1-Cub was only detected to interact with NubG-GPA1. Interaction between GPA1 and PI3K was a false positive result because PI3K-Cub was detected to interact with the empty NubG vector (data not shown). Therefore, I attempted to further confirm the interactions between GPA1 with GPA1, PLC1, PLC2 and PLDβ1 via *in vivo* co-immunoprecipitation methods.

Two independent *in vivo* co-immunoprecipitation methods were pursued to confirm the interactions between GPA1 and its candidate interactors. In these methods, I attempted to express the GPA1 candidate interacting proteins in tobacco leaves or Arabidopsis leaves respectively. For *in vivo* co-immunoprecipitation, FLAG tag was fused to either the N- or the C-terminus of each candidate GPA1 interacting protein (GPA1 or PLC1 or PLC2 or PLDβ1). For unknown reasons, the FLAG tag was not

successfully fused to PLDβ1 at that time. A construct containing each of the FLAG-tagged candidate proteins (FLAG-GPA1, GPA1-FLAG, FLAG-PLC1, PLC1-FLAG, FLAG-PLC2 or PLC2-FLAG) was used to infiltrate tobacco leaves (Nimchuk et al., 2000; Voinnet et al., 2003). The FLAG tagged GPA1, PLC1 or PLC2 were visualized by FLAG antibody on the western blot membrane, and tobacco leaves without infiltration was used as a negative control. Western blot assay showed that no protein bands were detected from the negative control, as expected, but two bands were detected from all infiltrated tobacco leaves (**Figure 5-2**). The molecular masses of these two bands (~70-80 kD) are similar to the molecular masses of PLC1 or PLC2, but are ~ 30 kD higher than that of GPA1.

The L-CFP-GPA1 construct, generated by inserting CFP between the 97$^{th}$ and 98$^{th}$ amino acid in GPA1 (Chen et al., 2003), was introduced into Col and *gpa1-4* background respectively to generate stable transgenic Arabidopsis plants. CFP signal was detected on western blot membranes using proteins extracted from five out of the 26 L-CFP-GPA1/*gpa1-4* transgenic plants (**Figure 5-3**) but not from any of 100 L-CFP-GPA1/Col transgenic plants for unknown reasons. The round leaf phenotype of *gpa1-4* was rescued in the five transgenic L-CFP-GPA1/*gpa1-4* plants. For *in vivo* co-immunoprecipitation, FLAG-tagged candidate GPA1 interacting proteins (GPA1 or PLC1 or PLC2) were transiently introduced into L-CFP-GPA1/*gpa1-4* transgenic plants using an *Agrobacterium*-mediated transient expression method (Lee and Yang, 2006). Unfortunately, FLAG signal could not be detected from the transfected Arabidopsis plants.

*In vitro* co-immunoprecipitation using recombinant proteins was not considered at the beginning because *in vivo* confirmation using plant proteins is the preferred choice. To continue this work, several strategies can be applied, e.g transient transfection of the FLAG-tagged GPA1 candidate interacting proteins into mesophyll cell protoplasts, or co-immunoprecipitate using recombinant proteins together with plant proteins.

**Results from TAP**

A 3 FLAG-3 HA-GPA1 construct was introduced into *gpa1-4* plants. RT-PCR and western blot were performed to evaluate the transcript and protein levels of 3FLAG-3HA-GPA1 (**Figure 5-4**). The transcriptional and translational products of 3FLAG-3HA-GPA1 were detected in two transgenic lines (T51 & T63) (**Figure 5-4 B-C**). However, the detected molecular weight of FLAG-GPA1 on the western blot membrane was 30 kD higher than predicted (**Figure 5-4 C**). At the same time, to further confirm the expression of the 3 FLAG-3 HA-GPA1, HA antibody from Sigma was also used for western blot assay. The result showed that this HA antibody is not a specific antibody to the HA tag since a band of similar molecular weight could be also detected from Col plants (**Figure 5-4D**). Unluckily, the round leaf phenotype of *gpa1-4* was not rescued in the 3 FLAG-3 HA-GPA1 transgenic plants (data not shown), and the FLAG-GPA1 band could not be detected in the second generation of the transgenic plants. This project was then terminated.

**Co-immunoprecipitation of GPA1 protein complexes**

Co-immunoprecipitation with GPA1 polyclonal antibody was performed using plasma membrane proteins from wild type and *gpa1* mutants. In this experiment, the *gpa1* mutant was the negative control, and GPA1 interaction partners were presumably co-immunoprecipitated with GPA1 antibody in WS but not in *gpa1* mutants. One protein was precipitated from wild type but not from the *gpa1* mutant (**Figure 5-5**).

The amount of protein in the weak band in **Figure 5-5** was not great enough to meet the sensitivity of MS identification and the band was not detected in other replicates. This low reproducibility may be due to the low abundance of GPA1 in Arabidopsis.

**Blue native 2D gel analysis of plasma membrane proteins**

Plasma membrane proteins were extracted from Col leaves using very mild conditions ($0^{o}$C and non-denaturing detergent) in order to preserve protein complexes in the solution. Plasma membrane proteins were resolved on native 2D gels, followed by western blot assay to confirm the presence of GPA1. Result from western blot assay suggested that the range of molecular weight of GPA1 complexes is from 100 kD to 300 kD, and four spots were detected on the western membrane (**Figure 5-6**). These results indicate that several GPA1 complexes may be present within the Arabidopsis plasma membrane. Unfortunately, upon staining with either silver or CBB method, bands were not detected on the second dimension gel at the corresponding position to the GPA1 bands on the western blot membrane (data not shown), indicating the protein abundance of GPA1 is below the detection limit.

**Discussion**

SUS has been applied successfully to evaluate candidate GPA1 interacting proteins including GCR1 (Pandey and Assmann, 2004), RGS1 (Chen et al., 2003), and AGB1 (Chen et al., 2006). GCR1 was used as a positive control and showed strong interaction with GPA1 in my SUS experiments, indicating SUS processes were correctly performed. Four GPA1 interacting candidates were identified via SUS methods. Unlucky, PLDα1 did not show strong interaction with GPA1 in SUS growth assay (no growth on 1 mM Met plates, data not shown), although PLDα1 was shown to be a GPA1 interacting partner by co-immunoprecipitation method (Zhao and Wang, 2004). This result suggests that 1 mM Met might be too strong to detect GPA1 candidate interacting proteins in SUS system (W. Frommer, personal communication). Therefore, all proteins which showed yeast growth on plates with 200 μM Met can be further studied.

Cub-GPA1 can only interact with NubG-GPA1, suggesting that free N and C termini might be required for the GPA1 homodimer formation. Given the fact that L-

CFP-GPA1 can rescue the round leaf phenotype of *gpa1* mutants (Wang et al., 2008) but FLAG-GPA1 cannot, we can conclude free N and C termini are also important for proper function of GPA1 in Arabidopsis because L-CFP-GPA1 construct contains free N and C termini (Chen et al., 2003), however the N terminus of GPA1 is blocked by the FLAG tag in the FLAG-GPA1 construct.

GPA1 is localized at the plasma membrane in Arabidopsis (Adjobo-Hermans et al., 2006; Wang et al., 2008). Therefore, total plasma membrane protein from Arabidopsis was used for co-immunoprecipitation and blue native 2D gel experiments. However, the low abundance of GPA1 makes it impossible to visualize GPA1 protein complexes on gels using gel staining methods, even though GPA1 signal can be visualized on the western blot membranes via GPA1 antibody.

In summary, four methods were attempted to identify GPA1 interacting proteins. Unfortunately, no GPA1 interacting protein with high confidence has been identified in this study due to either poor performance of antibodies or the scarceness of the target protein. Future work will be discussed in Chapter 6.

## Material and methods

### Construct generation and SUS procedure

Full length cDNA sequences of GPA1 and eighteen candidate interacting proteins (**Table 5-1**, AtVps34, KAT1, PLC1, PLC2, PLC3, PLC4, PLC5, PLC6, PLC7, PLC8, PLC9, PLDα1, PLDα4, PLDβ1, PLDγ1, PLDγ3, PLDδ1, PLDζ1) were cloned into Cub and NubG vectors. PCR products of PLDα2, PLDα3, PLDβ2, PLDδ2 and PLDζ2 were not detected possibly due to the low transcript levels of these genes in Arabidopsis. At the same time, full length cDNA clones of these genes were searched from the stock centers, but none of them was available from the SALK stock center at that time. aca agt ttg tac aaa aaa gca ggc tct cca acc acc and tcc gcc acc acc aac cac ttt gta caa gaa agc tgg gta were

linked to the forward and reverse primers respectively for each gene. Please see **Table 5-3** for primer information. In these vectors, Cub can only link to the C-termini of genes but NubG can link to both N-and C-termini of genes. All Cub vectors were transformed into AP4 yeast strain, and all NubG vectors were transformed into AP5 yeast strain using a yeast transformation kit according to the protocol provided by the manufacturer (BD Bioscience). After mating AP4 with AP5, the diploid cells were streaked out on SD plates with supplement of different concentrations of Met (0, 200 μM and 1 mM). At least three replicates of yeast transformations were performed.

**Transient transformation**

L-CFP-GPA1 was introduced into Col and *gpa1-4* plants to generate stable transgenic plants (L-CFP-GPA1/Col and L-CFP-GPA1/*gpa1-4*) via the floral-dip method (Curtis, 2004). Full length cDNAs of *GPA1*, *PLC1*, *PLC2* and *PLDβ1* (primers are same as in **Table 5-3**) were cloned into the FLAG-tagged Gateway binary vectors (pGW11 and pGW12) driven by the 35S promoter of *CaMV*. These binary vectors were introduced into *Agrobacteria* which were used to infiltrate tobacco leaves (Voinnet et al., 2003) or L-CFP-GPA1/*gpa1-4* transgenic plant leaves (Lee and Yang, 2006). L-CFP-GPA1 construct (Chen et al., 2003) was kindly provided by Dr. Shiyu Wang in Dr. Nina Fedoroff's lab.

**Generation of TAP construct and transgenic plants**

In order to introduce *GPA1* into the pJim19-HA-FLAG vector, *GPA1* primers were designed with a SpeI restriction site at the the 5' end of the primer and a SacI restriction site at the 3' end of the primer. Full length *GPA1* cDNA with two enzyme linkers was cloned into *E. coli*. After digestion and ligation, the 3 FLAG and 3 HA fusion were confirmed to be upstream of the *GPA1* by sequencing the construct. The 3 FLAG-3

HA-GPA1 construct was then introduced into *Agrobacteria* by transformation. Transgenic plants were generated via the floral-dip method (Curtis, 2004). The pJim19-HA-FLAG binary vector was kindly provided by Dr. Jianping Yang in Dr. Haiyang Wang's lab at the Boyce Thompson Institute.

**Co-immunoprecipitation steps**

Plasma membrane proteins were extracted using the same method as described in Chapter 3. All the following steps were performed at 4$^{o}$C in a cold room. Six hundred μg of plasma membrane proteins were first incubated with four μL GPA1 polyclonal antibody (gift of Dr. Alan Jones) for two hours to form GPA1-antibody complex, then incubated with 450 μL of Protein G Sepharose 4 Fast Flow (Amersham Biosciences) overnight at 4$^{o}$C in a cold room. On the second day, the Sepharose was pelleted via centrifugation of the sample at 18,000 g for 2 min and washed 3 times with 800 μL of IP buffer. The washed Sepharose was boiled in 50 μL of SDS sample buffer for 5 min, and supernatant was obtained by spinning the sample at 18,000 g for 2 min. The 50 μL supernatant was then separated via SDS-PAGE. The gel was finally stained by silver staining to visualize all the proteins co-precipitated by GPA1 antibody. IP buffer contains 30 mM Hepes/NaOH, pH 7.0, 150 mM NaCl, 10 mM EDTA-Na, 1 mM $MgCl_2$, 0.1 mM DTT, 1% (W/V) Triton X-100. SDS sample buffer was 62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% beta-mercaptoethanol.

**Blue native 2D running conditions**

All of the following steps were performed at 4$^{o}$C in a cold room. Isolated non-denatured plasma membrane proteins were loaded into the non-denaturing PAGE gel. Proteins could obtain negative charges from the Coomassie blue dye in the blue cathode buffer. The inner chamber was filled with the blue cathode buffer and the outer chamber

was filled with anode buffer. The running condition was set initially at 80 V until the protein bands reached the separating gel, then voltage was increased to 200 V. When the running-front was at the middle of the separating gel, the blue cathode buffer was replaced with the colorless cathode buffer. Electrophoresis was stopped when bands reached the bottom edge of the gel. Lanes from the first dimension were cut, then incubated in the SDS sample buffer for 15 min, and then applied to the second dimension gel. The SDS-PAGE was performed at 200 V for 45 min. Western blotting was performed after the gel from SDS-PAGE was incubated in a transfer buffer containing 0.1% SDS for ~10 min at room temperature. Cathode buffer was 50 mM Tricine, 15 mM Bis-tris, pH 7.0, anode buffer was prepared with 50 mM Bistris, pH 7.0. Blue cathode buffer was made by adding 0.02% Coomassie blue G 250 into the cathode buffer. Sample buffer was 10 mM Bis-tris (pH 7.0), 50 mM e-amino caproic acid, 0.5% Coomassie blue G 250. Transfer buffer was 25 mM Tris, 192 mM Glycine and 20% methanol.

## References

**Adjobo-Hermans, M.J., Goedhart, J., and Gadella, T.W., Jr.** (2006). Plant G protein heterotrimers require dual lipidation motifs of Gα and Gγ and do not dissociate upon activation. J Cell Sci **119,** 5087-5097.

**Assmann, S.M.** (2002). Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. Plant Cell **14 Suppl,** S355-373.

**Assmann, S.M.** (2004). Plant G proteins, phytohormones, and plasticity: three questions and a speculation. Sci STKE **2004,** re20.

**Bauer, A., and Kuster, B.** (2003). Affinity purification-mass spectrometry. Powerful tools for the characterization of protein complexes. Eur J Biochem **270,** 570-578.

**Berggard, T., Linse, S., and James, P.** (2007). Methods for the detection and analysis of protein-protein interactions. Proteomics **7,** 2833-2842.

**Camacho-Carvajal, M.M., Wollscheid, B., Aebersold, R., Steimle, V., and Schamel, W.W.A.** (2004). Two-dimensional blue native/SDS gel electrophoresis of multi-protein complexes from whole cellular lysates: A PROTEOMICS APPROACH. Mol Cell Proteomics **3,** 176-182.

**Chen, J.G., Willard, F.S., Huang, J., Liang, J., Chasse, S.A., Jones, A.M., and Siderovski, D.P.** (2003). A seven-transmembrane RGS protein that modulates plant cell proliferation. Science **301,** 1728-1731.

**Chen, J.G., Ullah, H., Temple, B., Liang, J., Guo, J., Alonso, J.M., Ecker, J.R., and Jones, A.M.** (2006). RACK1 mediates multiple hormone responsiveness and developmental processes in Arabidopsis. J Exp Bot **57,** 2697-2708.

**Curtis, I.S.** (2004). Production of transgenic crops by the floral-dip method. In Transgenic Plants: Methods and Protocols, pp. 103-109.

**Fan, L.M., Zhang, W., Chen, J.G., Taylor, J.P., Jones, A.M., and Assmann, S.M.** (2008). Abscisic acid regulation of guard-cell $K^+$ and anion channels in Gβ and RGS–deficient Arabidopsis lines. Proc Natl Acad Sci U S A.

**Feng, S., Shen, Y., Sullivan, J.A., Rubio, V., Xiong, Y., Sun, T.P., and Deng, X.W.** (2004). Arabidopsis CAND1, an unmodified CUL1-interacting protein, is involved in multiple developmental pathways controlled by ubiquitin/proteasome-mediated protein degradation. Plant Cell **16,** 1870-1882.

**Huang, J., Taylor, J.P., Chen, J.G., Uhrig, J.F., Schnell, D.J., Nakagawa, T., Korth, K.L., and Jones, A.M.** (2006). The plastid protein THYLAKOID FORMATION1 and the plasma membrane G-protein GPA1 interact in a novel sugar-signaling mechanism in Arabidopsis. Plant Cell **18,** 1226-1238.

**Hunt, L., Otterhag, L., Lee, J.C., Lasheen, T., Hunt, J., Seki, Shinozaki, K., Sommarin, M., Gilmour, D.J., Pical, C., and Gray, J.E.** (2004). Gene-specific expression and calcium activation of *Arabidopsis thaliana* phospholipase C isoforms. New Phytologist **162,** 643-654.

**Iyer, K., Burkle, L., Auerbach, D., Thaminy, S., Dinkel, M., Engels, K., and Stagljar, I.** (2005). Utilizing the split-ubiquitin membrane yeast two-hybrid system to identify protein-protein interactions of integral membrane proteins. Sci STKE **2005,** pl3.

**Johnsson, N., and Varshavsky, A.** (1994). Split ubiquitin as a sensor of protein interactions *in vivo*. Proc Natl Acad Sci U S A **91,** 10340-10344.

**Jones, A.M., and Assmann, S.M.** (2004). Plants: the latest model system for G-protein research. EMBO Rep **5,** 572-578.

**Lapik, Y.R., and Kaufman, L.S.** (2003). The Arabidopsis cupin domain protein AtPirin1 interacts with the G protein alpha-subunit GPA1 and regulates seed germination and early seedling development. Plant Cell **15,** 1578-1590.

**Lee, M.W., and Yang, Y.** (2006). Transient expression assay by agroinfiltration of leaves. In Arabidopsis Protocols, pp. 225-229.

**Lis, R.v., Atteia, A., Mendoza-Hernandez, G., and Gonzalez-Halphen, D.** (2003). Identification of novel mitochondrial protein components of *Chlamydomonas reinhardtii*. A proteomic approach. Plant Physiol **132,** 318-330.

**Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W.H., and Ma, L.** (2007). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science **315,** 1712-1716.

**McCudden, C.R., Hains, M.D., Kimple, R.J., Siderovski, D.P., and Willard, F.S.** (2005). G-protein signaling: back to the future. Cell Mol Life Sci **62,** 551-577.

**Mishra, G., Zhang, W., Deng, F., Zhao, J., and Wang, X.** (2006). A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science **312,** 264-266.

**Mueller-Roeber, B., and Pical, C.** (2002). Inositol phospholipid metabolism in Arabidopsis. Characterized and putative isoforms of inositol phospholipid kinase and phosphoinositide-specific phospholipase C. Plant Physiol **130,** 22-46.

**Nimchuk, Z., Marois, E., Kjemtrup, S., Leister, R.T., Katagiri, F., and Dangl, J.L.** (2000). Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from Pseudomonas syringae. Cell **101,** 353-363.

**Obrdlik, P., El-Bakkoury, M., Hamacher, T., Cappellaro, C., Vilarino, C., Fleischer, C., Ellerbrok, H., Kamuzinzi, R., Ledent, V., Blaudez, D., Sanders, D., Revuelta, J.L., Boles, E., Andre, B., and Frommer, W.B.** (2004). $K^+$ channel interactions detected by a genetic system optimized for systematic studies of membrane protein interactions. Proc Natl Acad Sci U S A **101,** 12242-12247.

**Pandey, S., and Assmann, S.M.** (2004). The Arabidopsis putative G protein-coupled receptor GCR1 interacts with the G protein $\alpha$ subunit GPA1 and regulates abscisic acid signaling. Plant Cell **16,** 1616-1632.

**Park, K.Y., Jung, J.Y., Park, J., Hwang, J.U., Kim, Y.W., Hwang, I., and Lee, Y.** (2003). A role for phosphatidylinositol 3-phosphate in abscisic acid-induced reactive oxygen species generation in guard cells. Plant Physiol **132,** 92-98.

**Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B.** (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods **24,** 218-229.

**Qin, C., and Wang, X.** (2002). The Arabidopsis phospholipase D family. Characterization of a calcium-independent and phosphatidylcholine-selective PLD ζ1 with distinct regulatory domains. Plant Physiol **128,** 1057-1068.

**Reisinger, V., and Eichacker, L.A.** (2007). How to analyze protein complexes by 2D blue native SDS-PAGE. Proteomics **7,** 6-16.

**Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B.** (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol **17,** 1030-1032.

**Rubio, V., Shen, Y., Saijo, Y., Liu, Y., Gusmaroli, G., Dinesh-Kumar, S.P., and Deng, X.W.** (2005). An alternative tandem affinity purification strategy applied to Arabidopsis protein complex isolation. Plant J **41,** 767-778.

**Schagger, H., and von Jagow, G.** (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem **199,** 223-231.

**Temple, B.R., and Jones, A.M.** (2007). The plant heterotrimeric G-protein complex. Annu Rev Plant Biol **58,** 249-266.

**Very, A.A., and Sentenac, H.** (2002). Cation channels in the Arabidopsis plasma membrane. Trends Plant Sci **7,** 168-175.

**Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D.** (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J **33,** 949-956.

**Wang, S., Narendra, S., and Fedoroff, N.** (2007). Heterotrimeric G protein signaling in the Arabidopsis unfolded protein response. Proc Natl Acad Sci U S A **104,** 3817-3822.

**Wang, S., Assmann, S.M., and Fedoroff, N.V.** (2008). Characterization of the Arabidopsis heterotrimeric G protein. J Biol Chem.

**Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M.** (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science **292,** 2070-2072.

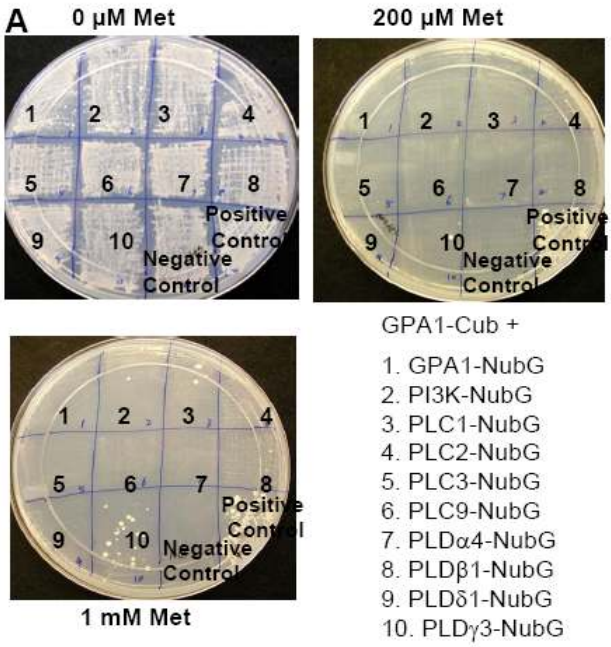
**Warpeha, K.M., Lateef, S.S., Lapik, Y., Anderson, M., Lee, B.-S., and Kaufman, L.S.** (2006). G-protein-coupled receptor 1, G-protein Gα-subunit 1, and prephenate dehydratase 1 are required for blue light-induced production of phenylalanine in etiolated Arabidopsis. Plant Physiol. **140,** 844-855.

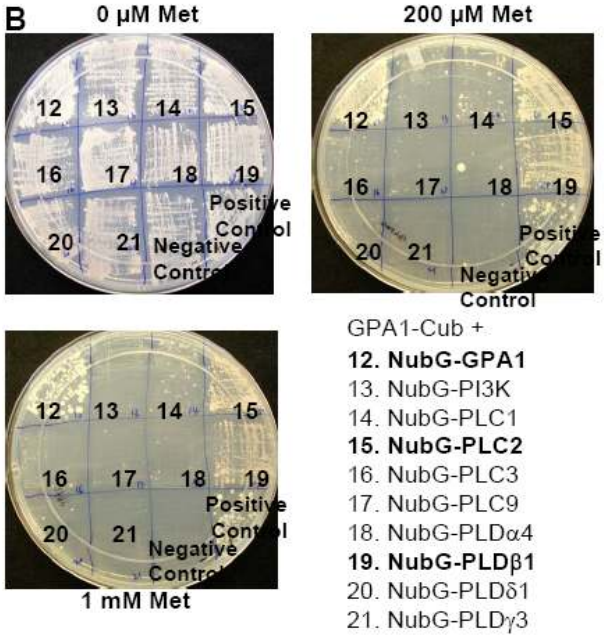
**Welters, P., Takegawa, K., Emr, S.D., and Chrispeels, M.J.** (1994). AtVPS34, a phosphatidylinositol 3-kinase of *Arabidopsis thaliana*, is an essential protein with homology to a calcium-dependent lipid binding domain. Proc Natl Acad Sci U S A **91,** 11398-11402.

**Witte, C.P., Noel, L.D., Gielbert, J., Parker, J.E., and Romeis, T.** (2004). Rapid one-step protein purification from plant material using the eight-amino acid StrepII epitope. Plant Mol Biol **55,** 135-147.

**Young, K.H.** (1998). Yeast two-hybrid: so many interactions, (in) so little time. Biol Reprod **58,** 302-311.

**Zhao, J., and Wang, X.** (2004). Arabidopsis phospholipase Dα1 interacts with the heterotrimeric G-protein alpha-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. J Biol Chem **279,** 1794-1800.


A
0 µM Met
200 µM Met
1 2 3 4
5 6 7 8
9 10
Positive Control
Negative Control
1 mM Met
GPA1-Cub +
1. GPA1-NubG
2. PI3K-NubG
3. PLC1-NubG
4. PLC2-NubG
5. PLC3-NubG
6. PLC9-NubG
7. PLDα4-NubG
8. PLDβ1-NubG
9. PLDδ1-NubG
10. PLDγ3-NubG


B
0 µM Met
200 µM Met
12 13 14 15
16 17 18 19
20 21
Positive Control
Negative Control
1 mM Met
GPA1-Cub +
12. NubG-GPA1
13. NubG-PI3K
14. NubG-PLC1
15. NubG-PLC2
16. NubG-PLC3
17. NubG-PLC9
18. NubG-PLDα4
19. NubG-PLDβ1
20. NubG-PLDδ1
21. NubG-PLDγ3

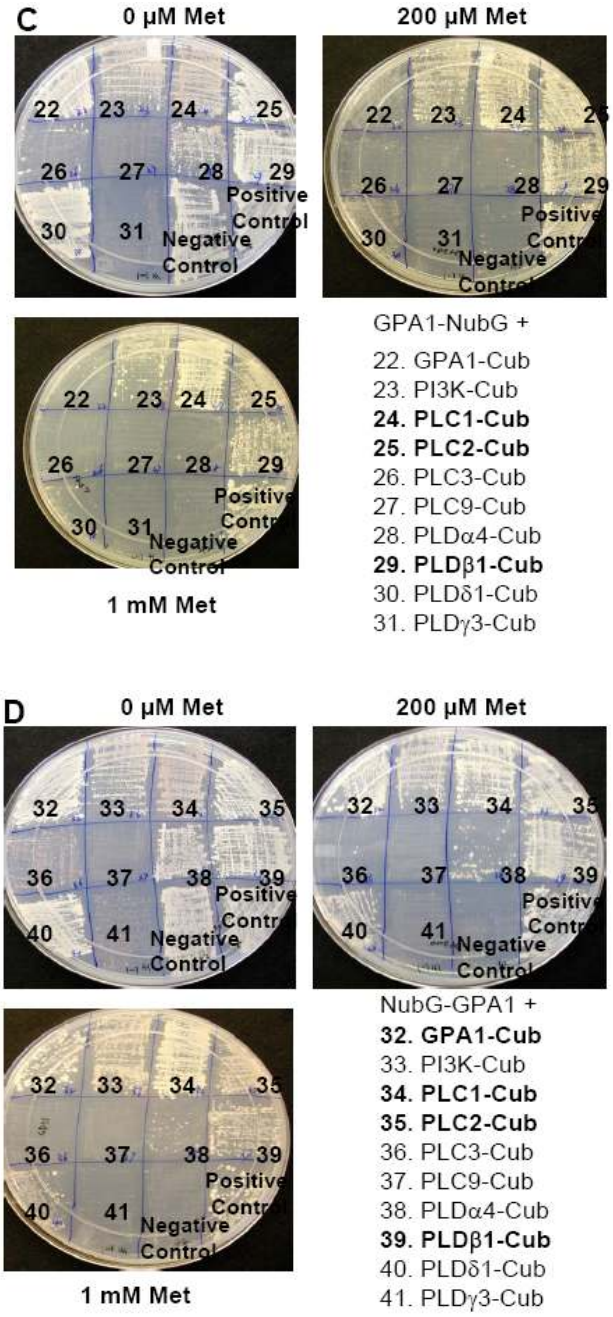



**Figure 5-1. Four candidate GPA1 interacting proteins, GPA1, PLC1, PLC2 and PLDβ1, were identified via yeast growth assay using SUS method.**

A. None of the C terminal NubG tagged proteins interacts with GPA1-Cub. B. NubG-GPA1, NubG-PLC2 and Ng-PLDβ1 interact with GPA1-Cub. C. GPA1-NubG interacts with PLC1-Cub, PLC2-Cub and PLDβ1-Cub. D. GPA1-Cub, PLC1-Cub, PLC2-Cub and PLDβ1-Cub interact with NubG-GPA1. Positive control: GPA1-Cub+P-NubG, Negative control: GPA1-Cub+Ng-GCR1.

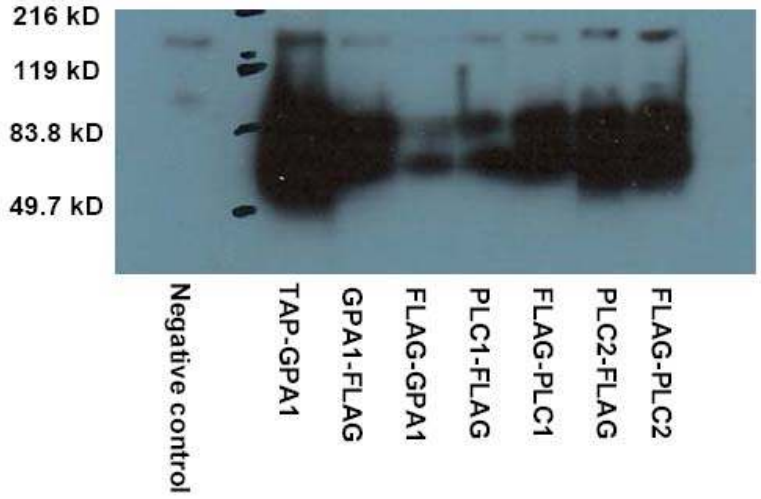



**Figure 5-2. Two bands were detected on the western membrane by FLAG antibody.**
Tobacco leaves without infiltration was used as the negative control.

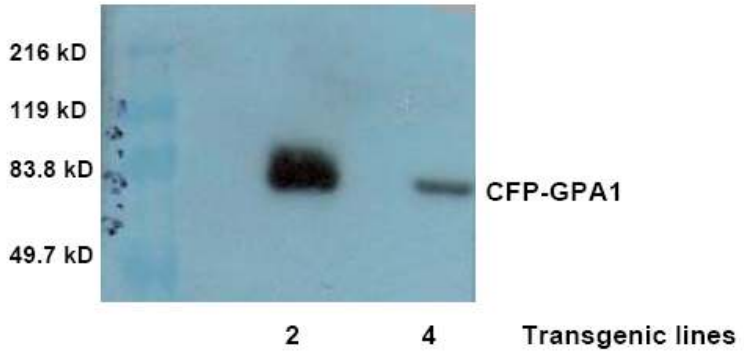



**Figure 5-3. CFP-GPA1 was successfully detected in transgenic line 2 and 4.**

Total protein was extracted from the transgenic Arabidopsis leaves. GFP antibody was used to detect the CFP-GPA1 protein.

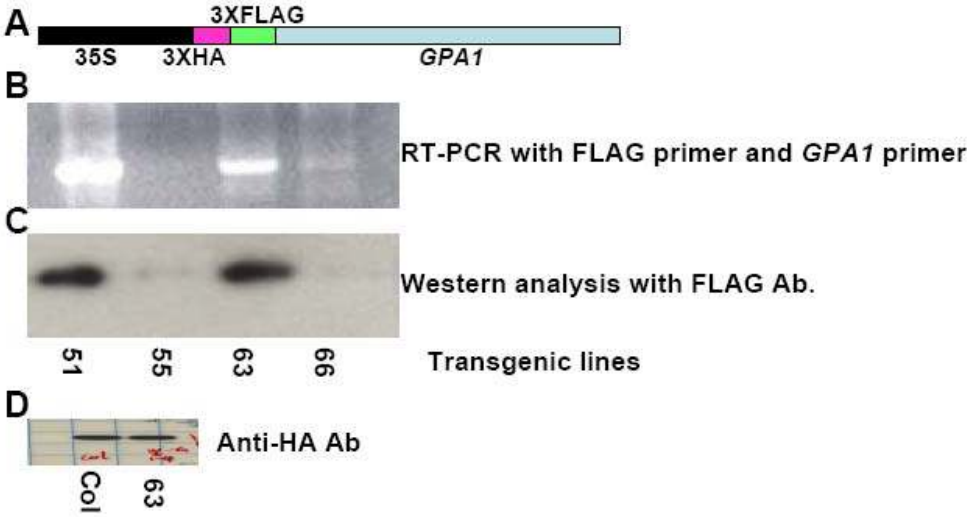



**Figure 5-4. Two successful transgenic lines, 51 and 63, were detected in the TAP transgenic plants.**

3 HA and 3 FLAG tags were linked at the of *GPA1* gene. The construct was driven by the 35S promoter of *CaMV*. B. FLAG-GPA1 was detected at transcriptional (B) and translational levels (C) in two transgenic lines, 51 & 63. D. HA antibody detected a non-specific band.

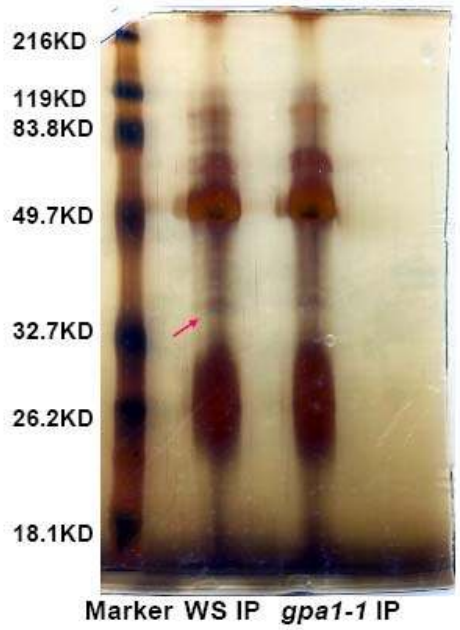



**Figure 5-5. One weak band was detected from WS but not from *gpa1-1* via co-immunoprecipitation with GPA1 antibody.**

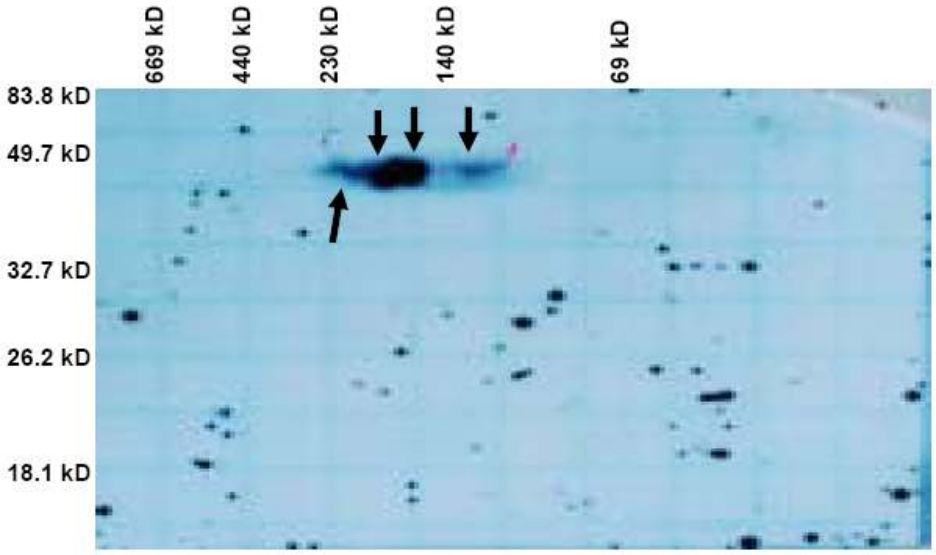



**Figure 5-6. Four GPA1 complexes were detected on the western blot membrane with GPA1 antibody.**

**Table 5-1**. **Putative GPA1 effectors in Arabidopsis. Bolded proteins have been tested via the SUS method in the present study.**

| | **Protein name and genomic locus** |
|---|---|
| Potassium channels | **KAT1 (At5g46240)**, KAT2 (At4g18290), KAT3 (AT4g32650), AKT1 (At2g26650), AKT2 (At4g22200), AKT5 (At4g32500), AKT6 (At2g25600) , KCO1 (AT5g55630), KCO2 (At5g46370), KCO3 (At5g46360), KCO4 (At1g02510), KCO5 (At4g01840), KCO6 (At4g18160),GORK(At5g37500), SKOR (AT3g02850), (Very and Sentenac, 2002) |
| PI3K | **AtVps34 (At1g60490)** (Hunt *et al*., 2004) |
| PLCs | **PLC1(At5g58670), PLC2 (At3g08510), PLC3 (At4g38530), PLC4 (At5g58700), PLC5 (At5g58690), PLC6 (At2g40116), PLC7 (At3g55940), PLC8 (At3g47290), PLC9 (At3g47220)** (Hunt *et al*., 2004) |
| PLDs | **PLDα1 (At3g15730)**, PLDα2 (At1g52570), PLDα3 (At5g25370), **PLDα4 (At1g55180)**, **PLDβ1 (At2g42010)**, PLDβ2 (At4g00240), **PLDγ1 (At4g11850)**, PLDγ 2 (At4g11830), **PLDγ 3(At4g11840)**, **PLDδ1 (At4g35790)**, **PLDζ1 (At3g16785)**, PLDζ 2(At3g05630) (Qin and Wang, 2002) |

**Table 5-2. Four proteins, GPA1, PLC1, PLC2 and PLDβ1, were identified to be GPA1 candidate interacting proteins.**

| At# | Name | 0 Met | 200 μM Met | 1 mM Met |
|---|---|---|---|---|
| At2g26300 | GPA1 | ++++ | ++ | ++ |
| At1g60490 | PI3K | ++++ | ++ | ++ |
| At5g58670 | PLC1 | ++++ | +++ | +++ |
| At3g08510 | PLC2 | ++++ | +++ | +++ |
| At4g38530 | PLC3 | ++ | - | - |
| At3g47220 | PLC9 | ++ | - | - |
| At1g55180 | PLDα4 | ++++ | + | + |
| At2g42010 | PLDβ1 | ++++ | +++ | +++ |
| At4g35790 | PLDδ1 | ++ | + | + |
| At4g11850 | PLDγ1 | ++++ | - | + |

+ means interaction. – means no interaction. The number of + indicates the strength of the interaction. ++++: strong interaction. +: weak interaction.

**Table 5-3**. **Sequence information of primers for *GPA1* and its candidate genes encoding putative GPA1 interacting proteins for SUS assay.**

| Primer name | Sequence |
|---|---|
| GPA1 Forward | ATGGGCTTACTCTGCAGTAGAA |
| GPA1 Reverse | TAAAAGGCCAGCCTCCAGTAA |
| $PI_3K$ Forward | ATGGGTGCGAACGAGTTTCGTTTCTT |
| $PI_3K$ Reverse | ACGCCAGTATTGAGCCCATCT |
| PLC1 Forward | ATGAAAGAATCATTCAAAGTGTGTTT |
| PLC1 Reverse | ACGAGGCTCCAAGACAAACCGCATG |
| PLC2 Forward | ATGTCGAAGCAAACGTACAAAGTG |
| PLC2 Reverse | CACAAACTCCACCTTCACGA |
| PLC3 Forward | ATGTCTTTCGATGAGCTTCTAA |
| PLC3 Reverse | ACGAAACGTATAAGGAGGATCCAAG |
| PLC4 Forward | ATGGAAGGAAAAAAAGAGATGGGTAGT |
| PLC4 Reverse | GACAAACTCGAAGCGCATAAGAAGC |
| PLC5 Forward | ATGGGGAGTTACAAA |
| PLC5 Reverse | AAGAAAGTGAAACCGCATGAGAAGT |
| PLC6 Forward | ATGGGGAAGGAGAAGAAAACAGAG |
| PLC6 Reverse | TTCGAAGATGAAACGCATAAGAAGC |
| PLC7 Forward | TCGAAGCAAACATACAAAGTCT |
| PLC7 Reverse | CACAAACTCCAACCGCACAAGAAG |
| PLC8 Forward | ATGTTAGTTACGAGGCGATGGGAAT |
| PLC8 Reverse | AGACCACTTAAAACGTGTGAGCA |
| PLC9 Forward | ATGGTGAATTTAAGAAAGAAGTTTGAGA |
| PLC9 Reverse | AGACCACTTAAAACGTGTGAGCA |
| PLDα1 Forward | ATG GCGCAGCATCTGTTGCACGGGACTT |
| PLDα1 Reverse | GGTTGTAAGGATTGGAGGCAGGTA |
| PLDα2 Forward | ATG GAAGAGTGTTTGTTACATG |
| PLDα2 Reverse | GGTTGTAAGAATTGGAGGCATGTAA |
| PLDα3 Forward | ATGACGGAGCAATTGCTGCTTCATGGAA |
| PLDα3 Reverse | AGAAGTAAGGATTGGAGGAAGATAA |
| PLDα4 Forward | ATGGAGCTTGAAGAACAGAAGAAGTACT |
| PLDα4 Reverse | GGTGGTTAGAACAGGAGGAAACATC |
| PLDβ1 Forward | ATGGATAATCACGGTCCTCGT |
| PLDβ1 Reverse | AATGGTTAGATTCTCTTGTATGG |
| PLDβ2 Forward | ATGGAGAATTATGGTTGGAA |
| PLDβ2 Reverse | GATAGTGAGATTCTCCTGTA |
| PLDγ1 Forward | ATGGCGTATCATCCGGCTTATA |
| PLDγ1 Reverse | TATGGTGAGGTTTTCTTGTAGTGCA |
| PLDγ2 Forward | ATGTCAATGGGAGGAGGGTCAAA |
| PLDγ2 Reverse | GATGGTGAGGTTTTCTTGTAG |
| PLDγ3 Forward | ATGGCGTATCATCCAGTTTATAACG |
| PLDγ3 Reverse | TATGGTGAGGTTTTCTTCTACTACA |
| PLDδ1 Forward | ATGGCGGAGAAAGTATCGGAGGACG |
| PLDδ1 Reverse | CGTGGTTAAAGTGTCAGGAAGAGCC |
| PLDζ1 Forward | ATGGCATCTGAGCAGTTGAT |
| PLDζ1 Reverse | TGGAAGACTTGAGGGGAGGCGTA |
| PLDζ2 Forward | ATGTCGACGGATAAATTACTACTTC |
| PLDζ2 Reverse | GTGGAAGACTTGAGGAGCAG |

# Chapter 6

# Retrospective and future work

Our guard cell proteomic study showed that TGG1 is the most abundant protein in Arabidopsis guard cells. Phenotypic studies of *tgg1* mutants led us to hypothesize a TGG1 working model in Arabidopsis guard cells (Chapter 2). Similarly, several stomatal movement phenotypes were detected in *ipgm* double mutants, but more experimental data are needed to further evaluate our hypotheses and elucidate the exact functions of glycolysis in guard cells. To evaluate our hypotheses about TGG1 and iPGMs functions in guard cells, future experiments are proposed in this chapter.

Guard cell specific and/or highly enriched proteins were discovered by comparison of our guard cell proteome to previously identified Arabidopsis proteomes (Chapter 2), and proteins quantitatively affected by ABA and/or GPA1 in guard cell were also detected by iTRAQ studies (Chapter 3). Therefore, functional characterization of these proteins in Arabidopsis guard cells will be helpful for understanding guard cell signaling. On the basis of the preliminary results (Chapter 5), future work is also proposed to identify GPA1 interacting proteins in this chapter.

**Functional characterization of TGG1 in Arabidopsis guard cells**

TGG1 was detected to be the most abundant protein in Arabidopsis guard cells (Chapter 2). Based on phenotypic analyses of *tgg1* mutants, we hypothesized that ABA induces the hydrolysis of glucosinolates by myrosinase via re-localization of glucosinolates to the cytosol from other intracellular compartments, e.g the vacuole, to inhibit $K^+_{in}$ channels in Arabidopsis guard cells (Chapter 2). Although localizations of glucosinolates in the vacuole (Kelly et al., 1998; Yan and Chen, 2007), and myrosinases in the cytosol have been reported (Luthy and Matile, 1984), the localizations of glucosinolates and myrosinase in guard cells are unknown. To study whether glucosinolates are localized in vacuoles, guard cell vacuoles can be isolated (Robert et al., 2007), and the content of glucosinolates in vacuoles can be measured (Barth and Jander, 2006). If glucosinolates do localize in vacuoles in guard cells, the levels of the glucosinolates in vacuoles can be studied before and after ABA treatment. To evaluate

the predicted cytosolic localization of TGG1 in Arabidopsis guard cells, GFP-tagged TGG1 can be generated and used to stably transfect Arabidopsis plants or transiently transfect Arabidopsis guard cells. Meanwhile, to evaluate whether this proposed novel pathway is unique in guard cells, the subcellular localization of TGG1 in Arabidopsis mesophyll cells can also be studied.

Previous study suggested that the aphid species, *M. persicae* and *B. brassicae*, could reproduce similarly on wild-type and *tgg* mutant plants, which might be due to little or no interaction between myrosinase and glucosinolates in Arabidopsis leaves (Barth and Jander, 2006). One experiment we can perform to assess this hypothesis is pretreatment of Arabidopsis leaves with ABA before aphid feeding. If ABA does induce hydrolysis of glucosinolates by myrosinase at the whole plant level, the reproductive growth of these aphid species will be much lower in wild-type compared to *tgg* mutants.

Electrophysiological results suggested that the hydrolyzed product(s) from glucosinolates are the functional molecule in ABA inhibition of $K^+_{in}$ channels in guard cells (Chapter 2). However, when stomatal movements were assessed by adding myrosinase or glucosinolates or a combination of myrosinase plus glucosinolates in the external incubation buffer, no effects were detected (data not shown). One explanation for this inconsistency is that both myrosinase and glucosinolates are too big to passively diffuse into Arabidopsis guard cells. One general property of the hydrolyzed products of glucosinolates is volatility (Yan and Chen, 2007), therefore, when glucosinolates and myrosinase are applied simultaneously in the incubation buffer in the future, the chamber should be sealed to prevent the evaporation of the hydrolyzed products.

**Functional characterization of iPGMs in Arabidopsis guard cells**

The *ipgm* double mutants are hyposensitive to blue light-promotion of stomatal opening, low-$CO_2$ induction of stomatal opening and ABA regulation of stomatal movements (Chapter 2), however, the mechanisms still remain to be discovered. Blue light induces malate accumulation in guard cell through glycolysis (Shimazaki et al.,

2007), and this process was impaired in a starch-deficient mutant, phosphoglucomutase mutant (Lasceve et al., 1997). Phosphoglucomutase transfers a phosphoryl group between glucose 1-phosphate and glucose 6-phosphate upstream of the glycolytic pathway. Therefore, we proposed that the blue light induction of malate accumulation in *ipgm* mutants is disrupted. To evaluate this hypothesis, malate concentration in guard cells in Col, *ipgm* single and double mutants under blue light should be measured.

Glucose generated by photosynthesis is hydrolyzed in the glycolysis pathway to produce energy. Therefore, glycolysis is positively related to photosynthetic activity. Since *igpm* double mutants are hyposensitive to ABA regulation of stomatal movements, we hypothesize that ABA negatively regulates glycolysis by reducing guard cell photosynthetic activity, then further reduces the 3-PGA concentration in guard cells (Mittelheuser and Steveninck, 1971; Gotow et al., 1988), and this ABA-inhibition of the 3-PGA production pathway is disrupted in *ipgm* double mutants. Twenty-one out of the 55 glycolytic enzymes, including iPGM1 and iPGM2, were detected by iTRAQ method in guard cells. However, none of the 21 glycolytic proteins was quantitatively affected by ABA in Arabidopsis guard cells. Therefore, to evaluate our hypothesis that glycolysis is inhibited by ABA, the iPGM enzyme activity in Arabidopsis guard cells can be measured (Westram et al., 2002; Bourgis et al., 2005) in Col and *ipgm* mutants before and after ABA treatment. The photosynthetic activity and 3-PGA concentration can also be measured in Col and *ipgm* single and double mutants. Photosynthesis in a single guard cell protoplast can be assessed by measurement of chlorophyll fluorescence using a microfluorometer (Goh et al., 1999) and 3-PGA concentration in guard cell protoplasts can be measured by a spectrometric method (Michal, 1984).

**Characterization of the novel GPA1 functions in Arabidopsis**

GPA1 was proposed to negatively regulate the photosynthetic rate, and positively regulate the concentration of the reactive oxygen species in Arabidopsis guard cells. To evaluate these hypotheses, Photosynthesis in Col and *gpa1-4* guard cells can be measured

and compared via a microfluorometer (Goh et al., 1999), and the concentration of ROS in Col and *gpa1-4* guard cell can be measured using 2′,7′-dichlorofluorescin diacetate ($H_2$DCF-DA) (Murata et al., 2001).

**Identification of GPA1 interacting proteins**

Several GPA1 candidate interacting proteins, GPA1, PLC1, PLC2, and PLDβ1, have been identified by SUS assay. Experiments should be performed to confirm the interactions between GPA1 and these candidate GPA1 interacting proteins. Recombinant GPA1-FLAG protein has been successfully expressed in *E. coli*. Candidate proteins can be linked with a tag (GST or His) and then expressed in *E .coli* to obtain recombinant proteins. Thereafter, *in vitro* co-immunoprecipitation can be performed to confirm interactions between GPA1 and the candidate GPA1 interacting proteins. Alternatively, the recombinant candidate interacting proteins can also be incubated together with proteins extracted from L-CFP-GPA1/*gpa1-4* transgenic plants for co-immunoprecipitation to confirm the interactions. For *in vivo* interaction, the construct containing GST- or His-tagged candidate proteins can be used to transiently transfect Arabidopsis mesophyll cell protoplasts (Yoo et al., 2007). Since GPA1 antibody is available, interactions between GPA1 and these candidate GPA1 interacting proteins can be studied by co-immunoprecipitation method. It was proposed in Chapter 5 that free N and C termini are important for homodimer formation of GPA1. To assess this hypothesis, a tag (e.g. His) can be fused to the N- or C- terminus of the GPA1 protein. Interaction between GPA1-FLAG and His-GPA1, or GPA1-FLAG and GPA-His can be studied. If interaction between GPA1-FLAG and His-GPA1 is detected, but not between GPA1-FLAG and GPA1-His, it indicates that free N- and C-termini are important for proper formation of GPA1.

Since low abundance of GPA1 hampers the ability to obtain definitive results from co-immunoprecipitation and blue native 2D experiments, homozygous L-CFP-GPA1/*gpa1-4* transgenic plants (**Figure 5-3**, line 2) will be greatly helpful for

identification of GPA1 interacting proteins. Proteins extracted from these transgenic plants can be further used for co-immunoprecipitation and blue native 2D experiments. GFP antibody can be used to pull down the GPA1 complex from the L-CFP-GPA1/*gpa1-4* transgenic plants.

**Functional characterization of the guard cell specific proteins**

Compared to previously identified proteomes from Arabidopsis, we discovered 71 guard cell specific (or highly enriched) proteins (Chapter 2). Five out of the 71 proteins, At1g73670 (MPK15), At3g18040 (MPK9), At4g28650 (leucine-rich repeat transmembrane protein kinase), At5g53320 (leucine-rich repeat transmembrane protein kinase) and At2g31083 (CLE7), are predicted to be signal transduction proteins by GO software (Chapter 2). MPKs (Wang et al., 2007) and leucine rich repeat transmembrane protein kinases (Osakabe et al., 2005; Shpak et al., 2005) have been demonstrated to be important for stomatal development and patterning in Arabidopsis. These five proteins will be good candidates for studying guard cell signaling and development. The ten unknown proteins out of these 71 proteins are also good candidates for future studies. T-DNA insertional mutants lacking any of these candidate proteins can be used for functional characterization of protein functions in Arabidopsis guard cells.

**References**

**Barth, C., and Jander, G.** (2006). Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. Plant J **46,** 549-562.

**Bourgis, F., Botha, F.C., Mani, S., Hiten, F.N., Rigden, D.J., and Verbruggen, N.** (2005). Characterization and functional investigation of an *Arabidopsis* cDNA encoding a homologue to the d-PGMase superfamily. J Exp Bot **56,** 1129-1142.

**Goh, C.H., Schreiber, U., and Hedrich, R.** (1999). New approach of monitoring changes in chlorophyll *a* fluorescence of single guard cells and protoplasts in response to physiological stimuli. Plant, Cell & Environ **22,** 1057-1070.

**Gotow, K., Taylor, S., and Zeiger, E.** (1988). Photosynthetic carbon fixation in guard cell protoplasts of *Vicia faba* L.: evidence from radiolabel experiments. Plant Physiol **86,** 700-705.

**Kelly, P.J., Bones, A., and Rossiter, J.T.** (1998). Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of *Brassica juncea*. Planta **206,** 370-377.

**Lasceve, G., Leymarie, J., and Vavasseur, A.** (1997). Alterations in light-induced stomatal opening in a starch-deficient mutant of *Arabidopsis thaliana* L deficient in chloroplast phosphoglucomutase activity. Plant Cell and Environ **20,** 350-358.

**Luthy. B. and Matile, Ph.** (1984). The mustard oil bomb: Rectified analysis of the subcellular organisation of the myrosinase system. Biochem. Physiol. Pflanzen **179**, 5-12.

**Michal, G.** (1984). D-Fructose 1,6-bisphosphate, dihydroxy-acetone phosphate and D-glyceraldehyde 3-phosphate. Methods of Enzymatic Analysis, 3rd edit. (Bergmeyer, H. U., ed.), vol. VI, pp. 342-350.

**Mittelheuser, C.J., and Steveninck, R.F.M.** (1971). Rapid action of abscisic acid on photosynthesis and stomatal resistance. Planta **97,** 83-86.

**Murata, Y., Pei, Z.M., Mori, I.C., and Schroeder, J.** (2001). Abscisic acid activation of plasma membrane $Ca^{2+}$ channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase 2C mutants. Plant Cell **13,** 2513-2523.

**Osakabe, Y., Maruyama, K., Seki, M., Satou, M., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2005). Leucine-rich repeat receptor-like kinase1 is a key membrane-bound regulator of abscisic acid early signaling in Arabidopsis. Plant Cell **17,** 1105-1119.

**Robert, S., Zouhar, J., Carter, C., and Raikhel, N.** (2007). Isolation of intact vacuoles from Arabidopsis rosette leaf-derived protoplasts. Nat Protoc **2,** 259-262.

**Shimazaki, K., Doi, M., Assmann, S.M., and Kinoshita, T.** (2007). Light regulation of stomatal movement. Annu Rev Plant Biol **58,** 219-247.

**Shpak, E.D., McAbee, J.M., Pillitteri, L.J., and Torii, K.U.** (2005). Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science **309,** 290-293.

**Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., and Zhang, S.** (2007). Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell **19,** 63-73.

**Westram, A., Lloyd, J.R., Roessner, U., Riesmeier, J.W., and Kossmann, J.** (2002). Increases of 3-phosphoglyceric acid in potato plants through antisense reduction of cytoplasmic phosphoglycerate mutase impairs photosynthesis and growth, but does not increase starch contents. Plant, Cell & Environ **25,** 1133-1143.

**Yan, X., and Chen, S.** (2007). Regulation of plant glucosinolate metabolism. Planta **226,** 1343-1352.

**Yoo, S.D., Cho, Y.H., and Sheen, J.** (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc **2,** 1565-1572.

# VITA

**Zhixin Zhao**

208 Mueller Labortary, The Pennsylvania State University
University Park, PA, 16802
Tel: (814)863-9578
Email: zuz101@psu.edu

**Education**

| | |
|---|---|
| 1994-1998 | B.S., Pomology, China Agricultural University, Beijing, China |
| 1998-2001 | M.S., Plant Physiology, China Agricultural University, Beijing, China |
| 2001-2008 | Ph.D. student, Plant Phsyiology, Penn State University, University Park, PA, USA |

**Technical Training:**

| | |
|---|---|
| 2004 May 22-23 | MALDI-TOF/MS: Fundamentals and Applications |
| 2007 June 2-3 | FTMS: Principles and Applications |

**Publications**

**Zhao Z**, Zhang W, Stanley B, Assmann SM. Functional proteomic studies of *Arabidopsis thaliana* guard cells uncover new signaling pathways in stomata. Submitted.

Pandey S, Perfus-Barbeoch L, Taylor J.P and **Zhao Z**. Plant Cell Signaling: *In Vivo* and -omics Approaches. J of Plant Growth Regulation. 2005 24(1):46-54

Fan LM, **Zhao Z** and Assmann SM. Guard cells: a dynamic signaling model. Curr Opin Plant Biol. 2004 7(5):537-46.

Zhang DP, Wu ZY, Li XY and **Zhao ZX**. Purification and identification of a 42-kilodalton abscisic acid-specific-binding protein from epidermis of broad bean leaves. Plant Physiol. 2002 128(2):714-25.